# Biocidal Effect of Alternating Current Technology on Planktonic Foodborne Pathogens

A Thesis
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I would like to especially thank my husband who supported me in going back to school, the start of which fell 10 days before our wedding. Through all the late nights studying and long days in the lab he helped and supported me.

## Dedication

This thesis is dedicated to those lab mates, both past and present, who have helped develop my love of microbiology.

## Abstract

Electric current has been demonstrated to inhibit growth of bacteria in solution. Alternating current has been shown to reduce populations at two frequencies; 150 kHz, which has been considered as a tumor-treating field (TTField), and 10 MHz as an antimicrobial field (AMField). The present study attempted to design an apparatus to determine the efficacy of an alternating current device, as well as combine this technology with antimicrobials to determine the combined effect on planktonic bacteria**.** This study found that low salt solutions, when exposed to ACF for long exposure times, 24 and 48 hours, and combined with 100 ppm of sodium hypochlorite, slowed bacterial growth when grown in Mueller Hinton Broth.

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# Chapter 1:
# Introduction

Foodborne pathogens have been a major concern as a preventable source of illness. According to the CDC, foodborne illness is responsible for 128,000 hospitalizations and roughly 3,000 deaths in the United States each year (CDC.gov). With growing concern to use green, non-chemical substitutes in food preparation, alternative solutions are being sought out as a way of inactivating bacteria in contaminated sources.

## Bacteria in the food industry:

According to the CDC, the most common foods to be associated with foodborne illness are raw foods of animal origin, filter feeding shellfish, and food products that combine products from multiple individual animals such as raw milk, raw eggs, or ground beef food products. Fruits and vegetables were more recently found to be a source for foodborne outbreaks. Many of these outbreaks were found to be caused by unsanitary wash water (CDC.gov).

Disinfection of wash process water is a critical step in decreasing cross contamination. However, studies have shown that there are many cases where chlorinated water on its own is not thoroughly effective in the reduction of pathogens, and thus allows for cross contamination during industrial processing (Zhang *et al*., 2009). Several of the main bacterial contributors for foodborne outbreaks are *Escherichia coli* O157:H7 and

*Salmonella enterica*, which are both Gram-negative rod shaped bacteria, and *Listeria monocytogenes*, which is a Gram-positive rod-shaped bacterium (CDC.gov).

## Electric current as an antimicrobial:

The use of electric current for the purpose of increasing antimicrobial efficacy has been investigated as a supplement to chemical practices in medical (Wellman *et al*., 1996, Caubet *et al*., 2004, Maadi *et al*., 2010, Kirson *et al.,* 2004, 2007, Giladi *et al.,* 2010), environmental (Wake *et al*., 2006, López-Gálvez *et al*., 2012), and industrial (Caubet *et al.*, 2004) settings for reducing bacterial populations. The use of electrical stimulation to affect bacterial growth was first reported by Rowley et al., (1974), over 40 years ago. Although the precise mechanism is still under investigation, research has shown that the application of different currents and frequencies can inhibit the growth of bacteria (Kohno *et al.,* 2000; Giladi *et al*., 2008, 2010; Maadi *et al*., 2010; Inhan-Garip *et al.,* 2011), help prevent adhesion of bacteria to surfaces (Shim *et al*., 2011; Van Der Borden *et al*., 2004a; Hong *et al*., 2008), and aid in the removal of established biofilm formations on surfaces (Van Der Borden *et al*., 2004, 2005). Electric currents have been shown to prevent bacterial attachment to electrically conductive surfaces where current potential has been applied.

Adhesion of cells is governed by three forces: electrostatic, electroosmotic, and electrophoretic (Poortinga *el al*., 2001). External forces, such as shear rate, have also been shown to be a factor in bacterial adhesion (Van der Borden *et al.,* 2004a; Hong *et*

*al*., 2008). Negative current was shown to have better prevention of bacterial adhesion compared to positive current **(**Shim *et al*., 2011).

Electric currents have also been shown to enhance efficacy when combined with antimicrobials to reduce bacterial populations both in solution as well as in biofilms (Blenkinsopp *et al*., 1992; Costerton *et al*., 1994; Wellman et al., 1996; Caubet *et al*., 2004; Giladi *et al*., 2008; Torgomyan *et al*., 2011; Torgomyan and Trchounian 2012; Mirazzi *et al*., 2015). However, problems have arisen in the consensus of standardizing the method and mode of application. Many factors must be taken into consideration such as voltage, current intensity, electrode use and material, and duration of treatment (Valle *et al*., 2007).

Diverse electric currents have been studied for their antimicrobial effectiveness. Electromagnetic current (Kohno *et al*., 2000; Inhan-Garip *et al*., 2011; Fojt *et al*., 2009), direct current (Liu *et al*., 1997; Maadi et al., 2010), radio frequency electric current (Caubet *et al*., 2004) and alternating current (Pareilleux and Sicard 1970; Caubet *et al*., 2004; Kirson *et al*., 2004, 2007; Maadi *et al*., 2010; Giladi *et al*., 2008, 2010) have all been investigated at varying frequencies. Each current has been investigated to determine the optimal frequency to achieve bacterial inactivation.

## Electromagnetic current and the generation of electromagnetic fields:

Electromagnetic current has been examined using the generation of magnetic fields. This has been found to be bacteriostatic, rather than having a bactericidal effect. In a study

conducted by Kohno *et al*., (2000), the quantity of viable bacteria colonies after decreased according to the strength of the magnetic field. Bacteria were subjected to static magnetic fields of 30, 60, 80 and 120 mT (milliTesla) under both aerobic and anaerobic conditions in a 10nM Tris-HCl buffer. A ferrite plate delivered the magnetic current. It was found that bacterial growth was inhibited when bacteria were kept under anaerobic conditions and not aerobic conditions. This suggests there are large differences in the effect that the magnetic fields have on bacteria, contingent on the presence or absence of dissolved oxygen (Kohno *et al*., 2000).

Extremely low frequency electromagnetic fields of 50Hz, at 0.5 mT, exposed for 6 hours at 37°C were shown to decrease growth rate and induce morphological changes to both Gram-negative and Gram-positive bacteria. In *E. coli,* observations were made of disintegration of the cell wall, extrusion of the contents of the cytoplasm, and cell wall retraction of the cytoplasmic membrane. It was suggested that the magnetic field effect is maximized in the first hours of exposure, then decreases over time, implying that there is an adaptive response by the cells (Inhan-Garip *et al*., 2011). Previous studies have shown a decreased ability for bacteria to form colonies with the increase of magnetic field intensity as well as exposure time up to 120 minutes (Strasak *et al*., 2002, Fojt *et al*., 2004, El-Sayed *et al*., 2006).

In an experiment performed by Fotj *et al.,* (2004), *E. coli* strain K12 was exposed to SMF of 10 mT at 50 Hz for 60 minutes in either Petri dishes or on microscopic slides. Other

studies investigated the effect of 6 mT SMF on the induction of cell shape, cell surface and cytoskeleton on cells when exposed for 24 or 48 hours (Dini and Abbro 2005). It was found that apoptosis was increased in certain cells up to 20% after 24 hours of continuous exposure to 6 mT SMF.

**Direct current:**

Direct current (DC) has also been investigated, comparing the efficacy of both anodic and cathodic current. In one study, it was shown that the cathodic current had the most effect on bacterial inhibition (Shim *et al*., 2011). In contrast, Maadi *et al*., (2010) showed no statistical difference between electrode polarities, but did see a difference in growth around the anode due to the presence of toxic electrochemical products. Shim *et al*., (2011) used 10 uA DC, applied for 16h, which produced a zone of inhibition around the cathode under aerobic conditions, but not under anaerobic conditions, when an indium tin oxide film electrode was used. DC has also been shown to reduce bacterial adhesion on surfaces by ~80% when negative current was applied, but not significantly when exposed to positive current (Shim *et al*., 2011). Varying amperages, as well as the material of the electrode, have been shown to alter the effects on bacteria. In one experiment, 20 and 40 mA inhibited the growth of *E. coli* when graphite electrodes were used. However, 5 mA was enough to inhibit growth when copper electrodes were used on *E. coli* (Valle *et al*., 2007).

The proposed explanations for the antimicrobial properties of the direct current bioelectric effect are based on the modification of pH, transport of ions between the

electrodes or the production of additional biocides by electrolysis (Stoodley *et al*., 1997, Stewart *et al*., 1999, Wattanakaroon *et al*., 2000). Direct current has been associated with the production of $H_2O_2$ at the cathode side and chlorine production in the anode side (Liu *et al*., 1997), leading to the potential source of efficacy of the current.

**Radio frequency electric current:**

In contrast to the direct current bioelectric effect, radio frequency electric current, which produces a similar effect as alternating current, does not take present ions and transport them in the surrounding liquid, nor does it create new ions in the liquid at a 10MHz frequency and a low intensity. Radio frequency electric current does not produce electroporation effects or free oxygen or other electrolytic substances. It also does not produce a major heating effect (Caubet *et al*., 2004). Therefore, these factors cannot be contributing to the antimicrobial effect of the radio frequency electric current and liquid substrate on the bacterial cell.

**Alternating current:**

Alternating current has been used both *in vitro* (Giladi *et al*., 2008; Maadi *et al*., 2010, Mirzaii *et al*., 2015) and *in vivo* (Kirson *et al*., 2004, 2007, Giladi *et al*., 2010) as a means for inactivation of bacteria (Giladi *et al*., 2008, 2010, Maadi *et al*., 2010, Mirzaii *et al*., 2015) and disruption of cancer cell replication (Kirson *et al*., 2004, 2007). In studies performed on cancerous tumor cells, two optimal frequencies have been identified. An alternating current of 10 MHz has been described as creating an antimicrobial field (AMfields) (Caubet *et al*., 2004, Giladi *et al*., 2008), while a frequency of 150 kHz is considered a tumor-treating field (TTfields) (Kirson *et al*., 2004, 2007). There has been

no evidence of bacterial strains acquiring resistance to the inhibitory effect of the alternating fields (Giladi *et al*., 2008).

The inhibitory effect of TTfields has been attributed to two distinct mechanisms. First, interface with the formation of mitotic spindle microtubules, second, physical destruction of cells during cleavage. Both mechanisms are greatly dependent on the orientation of the axis during mitosis versus the field vectors (Kirson *et al*., 2004). A study performed by Kirson *et al*., (2004) demonstrated that an alternating electric field of 100 to 200 kHz interfered with the orientation of the spindle microtubules and the polymerization-depolymerization development involved in the chromosome separation process. Dividing cells that were affected by the electric current were also found to be oriented in the direction of the applied electric field. Therefore it was deduced from the study that the efficacy of TTfields must be a function of the angle between the field and the axis of division. When the two are parallel, the effect is maximal and when one is perpendicular to the other, it must be minimal (Kirson *et al*., 2004). This mechanism has been further studied in the application of AMfields in the reduction of bacteria (Giladi *et al*., 2008, Giladi *et al*., 2010).

Frequency dependent inhibition of bacterial growth has been shown to relate to the effect of the alternating electric fields on the enzyme-substrate reaction equilibrium (Robertson and Astumian 1990, Giladi *et al*., 2008). This model suggests the electric charge distribution on some enzymes fluctuates with the conformational changes associated with

enzyme-substrate interaction. This may be due to the alternating current affecting molecular charge distributions, which could interfere with enzymatic reactions. The effect is expected to be larger for membrane enzymes, due to the membrane hindering the enzyme from rotating and escaping the effect of the electric field, as well as the fact the electric field is amplified in the membrane (Giladi *et al*., 2008). If essential enzymes are affected by the electric fields, exposure of these enzymes to certain frequencies may inhibit bacterial growth by depleting the cell of the enzymes' products.

The combination of AMfields and antibiotics was found to produce an additive efficacy against biofilms and bacteria in solution (Caubet *et al*., 2004, Mirzaii *et al*., 2015). In a study conducted by Kirson *et al*., (2004), insulated electrodes were used to control for the generation of free radicals and to protect against the production of $H_2O_2$.

**Electrochemical effect:**

Electric currents have been used in combination with antimicrobials to achieve electrochemical inactivation of microbes. This can increase the efficacy of both the chosen electric current and the chemical for the inactivation of bacteria (Liu *et al*., 1997). It has been suggested that the electric current by itself is not able to inactivate the bacteria in a biofilm, but rather is able to act synergistically with biocides (Costerton *et al*., 1994).

The material composition of the electrode used to deliver the electric current has been shown to have different impacts on the level of the antimicrobial effectiveness of the electric current (Rosenberg *et al*., 1965, Davis *et al*. 1991, Kohno *et al*. 2000, Jeong *et al*.

2007, Valle *et al*. 2007, Jeong *et al*. 2009), as well as the generation of reactive oxygen species (ROS) (Jeong *et al*. 2009, López-Gálvez *et al*. 2012). The most common ROS are those produced by the oxidation of water molecules, including $^{\bullet}OH$, $O_3$ and $H_2O_2$. As described in Maadi *et al*., (2010), the anode side is responsible for the generation of free radicals when using direct current. This could result in the inactivation of the microbes depending on the duration of exchange intervals of the current (1 min and 1s) (Seok *et al*., 2008).

Previous studies applied electrodes that were not insulated, resulting in the generation of $H_2O_2$ and other oxidants (Liu *et al*., 1997, Jeong *et al*., 2009), as well as the addition of heat produced by the electric current (Giladi *et al*., 2010). This was concluded to have contributed to the biocidal effect, rather than being solely the result of the current itself (Giladi *et al*., 2010). Production of $H_2O_2$ at the surface of the electrode was found to enhance the activity of the electric current (Liu *et al*., 1997). Subsequent studies applied insulated electrodes to control for the generation of reactive oxygen species (Kirson *et al*., 2004, Giladi *et al*., 2008).

## Commercialization of alternating current technology:

Several companies have attempted to develop devices that would utilize electric current as a means of removing particles and conditioning water to help alleviate the need for harsh chemicals. One such company has applied this technology not only to condition water, but also has extended the technology to reduce bacterial populations using electric current.

The alternating current frequency (ACF) technology used in this device was created originally for the application of scale removal in pipes (Stephanini 1999). However, it has also been preliminarily shown to prevent, reduce and remove biofilm and bacterial counts in solution with prolonged application. This alternating current technology utilizes a ferrite ring, which provides a core with both a primary and secondary wiring to deliver an alternating current with a frequency range of 200 to 500 kHz. This frequency has a sinusoidally varying amplitude, which diminishes from a maximum value to zero. This oscillating sine wave of an induced alternating current signal causes the negatively and positively charged ions to form suspended clusters in the water, and prevents them from adhering to rough surfaces **(**Hydroflow-usa.com**).** The ferrite ring is secured over a pipe containing flowing water. Once the signal is initiated, the water inside the pipe acts as a conductor that carries the induced electric signal throughout the water system (Hydroflow-usa.com). An electromagnetic field is generated and is directly proportional to the condition of the piping structure and the amount of scale within the system (Stephanini 1999).

## Effect of Suspension Solution in combination with electric current on Bacteria:

Varying solutions have been used when studying the effect of electric current on bacterial cells. Synthetic urine has been added to investigate the iontophoretic killing of bacteria in fluid (Davis *et al*., 1989). NaCl concentrations have been investigated to determine the

free chlorine produced when added to tap water, resulting in bacterial reductions of 5 $\log_{10}$ cfu/ml faster than using tap water alone (Lopez-Galvez *et al*., 2012).

Different ionic strengths have been shown to aid in flocculation of activated sludge where bacteria may be trapped and settle out of solution. KCl and $CaCl_2$ were used at different ionic strengths. It was found that, at increasing concentrations of electrolytes, stability of flocs increased (Zita and Hermansson 1994). The mode of action for this technology uses this flocculation of charged ions as a way of filtering out particles from treated water systems. Ions become charged when passing through the ferrite ring, turbulence in the system mixes particles and creates flocs, filters then remove flocs from water, as shown in Figure 1.1 (Hydroflow-usa.com).

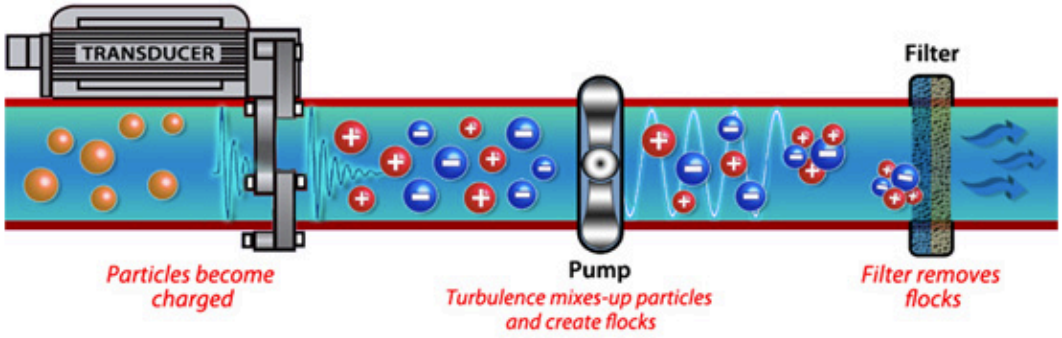


**Figure 1.1. Hydroflow proposed mode of action on particles suspended in solution.** (Source: Hydroflow-usa.com)

In this study we chose to focus not on the filtering effects of the alternating current technology, but rather on the effect of the alternating current frequency on planktonic bacterial growth, both in static and flow thru systems. Variables were chosen based on the frequency, circulation, and volume, as well as the suspension solution used to house the bacteria, to determine the impact on the overall efficacy of the alternating current. In

the first section (Chapter 2), methods were designed to investigate the overall log reduction of pure cultures in systems of various solution, volume and material that were subjected to different current frequencies. While the second section (Chapter 3) focuses on the effect of the alternating current on bacteria in a static system and the resulting effect on susceptibility when exposed to varying levels of antimicrobials.

# Chapter 2:

# Development of a Laboratory Apparatus to Simulate Field Studies to Determine the Biocidal Efficacy of an Alternating Current Technology

## Introduction:

For the purposes of this study we looked at the effect of the different alternating current frequency units on bacteria related to the food industry. One challenge that arose when choosing a method to investigate the efficacy of the alternating current device was determining the appropriate scale. Previous studies contracted by this company had reported a difficulty reproducing results seen in the field. Small scale studies utilized a few liters to 50 liters of water in flow thru systems. Investigations into the literature could not provide concrete solutions, as the scale of the experiments performed was much smaller (milliliters) than what was thought to represent an applicable field type situation.

Based on previous studies done in the field (Leung, Ivan 2011; Li, Jeremy 2011; Wang and Kelso 2013), ACF units are thought to have biocide capabilities. This is based on a hypothesis that a bacterial cell passing through the alternating current signal becomes charged, attracting water molecules to the bacteria, forming a layer of pure water free of ions. Osmosis would then draw the pure water thru the membrane, resulting in osmotic pressure building up in the cell, causing it to burst and causing cell death, as shown in

Figures 2.1 (Hydroflow-usa.com). Though there are no studies proving the hypothesis of this application, studies performed in the field have suggested that the alternating current technology may provide biocidal activity.

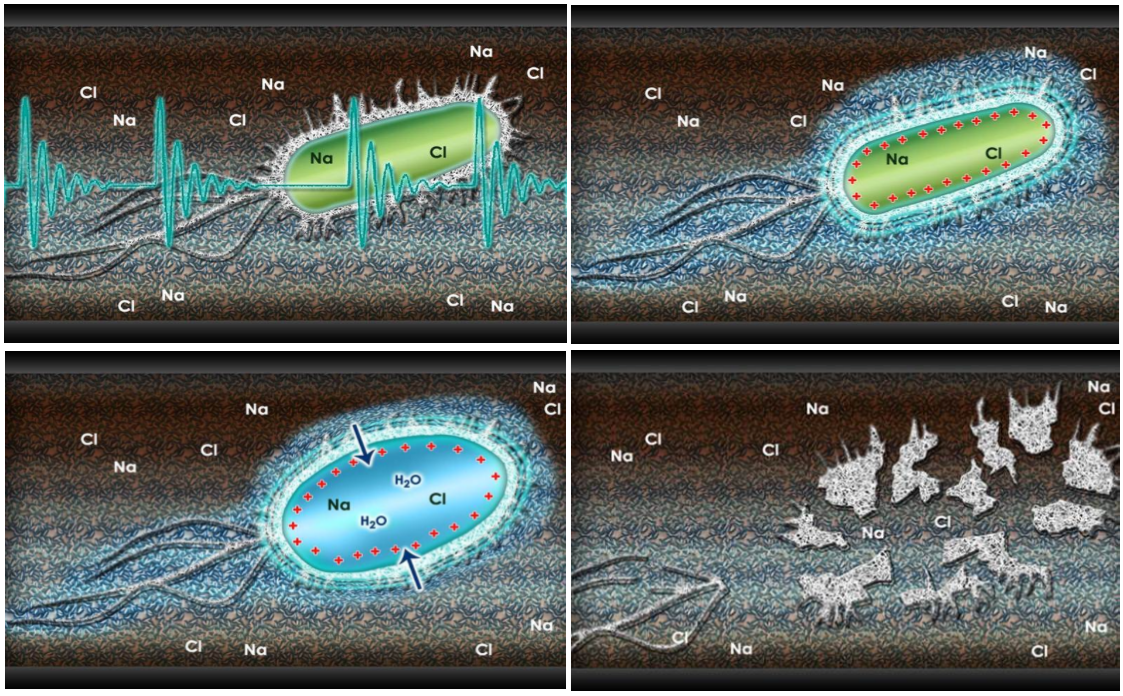


**Figure 2.1. Proposed mode of action of Hydroflow alternating current frequency on bacterial cells by polarity change and osmotic pressure. (Source: Hydroflow-usa.com)**

We investigated the use of alternating current as a biocide for planktonic bacteria in varying solutions, including different salts and growth promoters. Examination was done into the effect on both a Gram positive and Gram negative foodborne pathogen as a way to investigate the difference in the effect of the applied current on different bacteria, as shown in the work of Fotj *et al*., (2007). This study showed that a magnetic field of 10 mT and 50 Hz for 24 min caused a 20% decrease in cfu/ml in Gram-positive bacteria

with spherical shape and a 30-40% decrease in cfu/ml of Gram-negative bacteria with a rod shape.

Alternating Current Frequency (ACF) technology in a lab setting:

Studies have been done using alternating current devices in both lab and field settings. Studies performed in the lab have looked at *Legionella pneumophila* SG1, *Staphylococcus aureus* AACC 6538, and *Escherichia coli* AACC 8739. Each of four studies contracted by the developing company were performed using approximately $10^5$cfu/ml (colony forming unit per milliliter) of their respective bacteria. Three of the studies used normal tap water at different volumes as the liquid substrate to be circulated (Leung and Ivan 2011; Li, Jeremy 2011). The fourth study used "Test Water" prepared from laboratory reagent water, as well as physiological saline solution of 0.85% NaCl in the Test Water, as two separate testing variables (Wang and Kelso 2013). All studies circulated water exposed to the alternating current device for one hour. All four studies reported a 92.9%-99.99% reduction of bacteria after one hour after starting concentrations contained either $10^4$ cfu/ml or $10^6$ cfu/ml (Leung, Ivan 2011; Li, Jeremy 2011; Wang and Kelso 2013).

Exposure lengths and experimental design were chosen based on previous studies contracted by Hydropath, as well as literature investigating electric current that was done both *in vitro* and *in vivo*. (Hydropath lab report unpublished; Mirzaii *et al*., 2015; Giladi *et al*. 2008; Shim *et al.,* 2011; Huang *et al*., 2015)

The objective of this study was to determine whether the alternating current frequency (ACF) does in fact demonstrate biocidal effects under controlled conditions. This was investigated using different concentrations of *Salmonella enterica*, *Escherichia coli* O157:H7 and *Listeria monocytogenes,* both in static and flow systems. For the purposes of this study, we looked only at the effect of the alternating current on the planktonic populations in solution.

## Materials and Methods:

Culture Conditions:

Bacterial strains of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*, obtained from the University of Minnesota culture collections of environmental isolates, were grown up in Bacto™ Tryptic Soy Broth Soybean-Casein Digest Medium made by BD from freezer stocks kept in glycerol. Cultures were serially transferred twice from freezer stock and grown overnight ~ 18 hours in TSB at 37°C. Final concentrations of overnight cultures were approximately $10^{\wedge 9}$ cfu/ml. Cultures were serially diluted into 0.9% saline for inoculation of each experiment.

Pond water inoculum:

Pond water was obtained from Ramsey County on September 10$^{th}$, 2015. Normal flora from the pond water was used to test aerobic bacteria.

Microbial analysis:

3M® Aerobic Plate Count (APC) were used for bacterial growth and isolation. For sample enumeration, 1ml of sample was pipetted out of the test sample and serially diluted 1:10 into standard sterile saline dilution broth (0.9% saline). Samples were diluted to a countable range and plated on APC Petrifilm. Plates were incubated at 37°C for 48 hours and colony unit determined.

Physiochemistry Analysis:

Water analysis was conducted using an Orion A111 pH meter with a standard gel electrode, a pHep, manufactured by Hanna. Conductivity was taken using a Granger General Conductivity meter CO-502. ORP was measured using ORP meters ORPtestr10, Orion A111 pH meter with a standard gel electrode. Free Available Chlorine (FAC) test strips were purchased from LaMatte 4250 BJ. Hardness was measured using hardness strips 480008 from Waterworks. Electric Frequency was measured using an Omon oscilloscope model HDS1021M.

At each time point, readings were taken to measure pH, ORP, temperature, conductivity and free available chlorine from each system.

Alternating Current Model Units:

Three different alternating current model HS48 units: serial number 3105420112, serial number GMA-264785 01/11, serial number GMA-264973 1812, and a model 60i serial number GMA – 14540 3714.

Apparatus Equipment:

A stainless steel beaker 2 ¼" diameter, 5 ½" height, 2mm thick, as well as a 250ml glass Erlenmeyer flask. 3/4" galvanized steel pipe and galvanized steel joints. Corning PC-420D stir bar with magnetic stir bars. Plastic tubing of varying length attached to an Everbilt Non-Submersible Transfer Pump with a 360 gallon per hour flow rate 1/10 HP, or attached to a National Geographic CF30 Aquarium Canister Filter pump with a 132 gallons per hour flow rate. Finally, a 25L plastic carboy holding tank used in the circulation process.

Reagents:

Salt reagents sodium citrate lab grade granular and, potassium citrate monohydrate P218-500 purchased from Fisher Scientific. Calcium citrate tribasic tetrahydrate $Ca_3(C_6H_5O_7) \cdot 4H_2O$ was purchased from Acros Organics. Magnesium chloride 6-Hydrate crystal purchased from Baker Analyzed. D-Glucose from Teknova and NaCl from Morton.

Experimental Setup:

**Antimicrobial properties of HS48 ACF on *S. enterica* and *E. coli* O157:H7, inoculated into tap water in a static system for 20 minutes**

A unit model HS48 was placed over a stainless steel container (2 ¼" diameter, 5 ½" height, and 2mm thick), filled with 200ml of either tap water or deionized water. The alternating current device was placed on a small box to keep the unit from touching the bench top, while keeping the ferrite ring below the liquid line in the beaker (as shown in Figure 2.2)


**Figure 2.2. Stainless steel beaker in a static system with model HS48.**

***Antimicrobial properties of HS48ACF on L. monocytogenes, S. enteria, and E. coli O157:H7, inoculated into either tap water or deionized water in an agitated system for 60 minutes***

A unit model HS48 was placed over a stainless steel container (2 ¼" diameter, 5 ½" height, and 2mm thick) filled with 100ml of either tap water or deionized water. A magnetic stir bar was added to the stainless steel container. The container was placed on a Corning PC-420D stir plate at 300rpm. A small box was used to prop the device so that

it did not rest on the stir plate but allowed the ferrite ring to remain below the liquid line in the container, as shown in Figure 2.2.

***Effect of alternating current technology on water physiochemistry with the addition of 0.9% NaCl***

Two pieces of plastic tubing, 20 ½" and 9 ½", were connected by a brass adapter FGH x Barb on either side of an Everbilt Non-Submersible Transfer Pump. A 12" x ¾" galvanized steel pipe was connected by joint to a 3"x ¾" pipe. Two elbow connectors were attached to either end of the combined pipes. The elbow connected to the 12" pipe was joined to a ½" x ¾" nylon barb x MIP adapter. This was connected to a 20 ½" plastic tubing, which was connected to the Everbilt pump attached to the output side of the pump by a brass adapter FGH x Barb. The opposite elbow was connected to a 3" nipple leading into a 12 ¾" x 10 ½" x 4" 6.7 qt stainless steel holding pan. A second piping set was constructed for the side leading into the pump. This was constructed similar to the first, but only exchanging the 3" nipple for an 8" x ¾" piping closer to the pump. A HS48 device was secured over the steel pipe leading into the pump. The final setup can be seen in Figure 2.3. 2L of either 0.9% sterile saline solution or deionized water was measured into the stainless steel holding pan for testing.

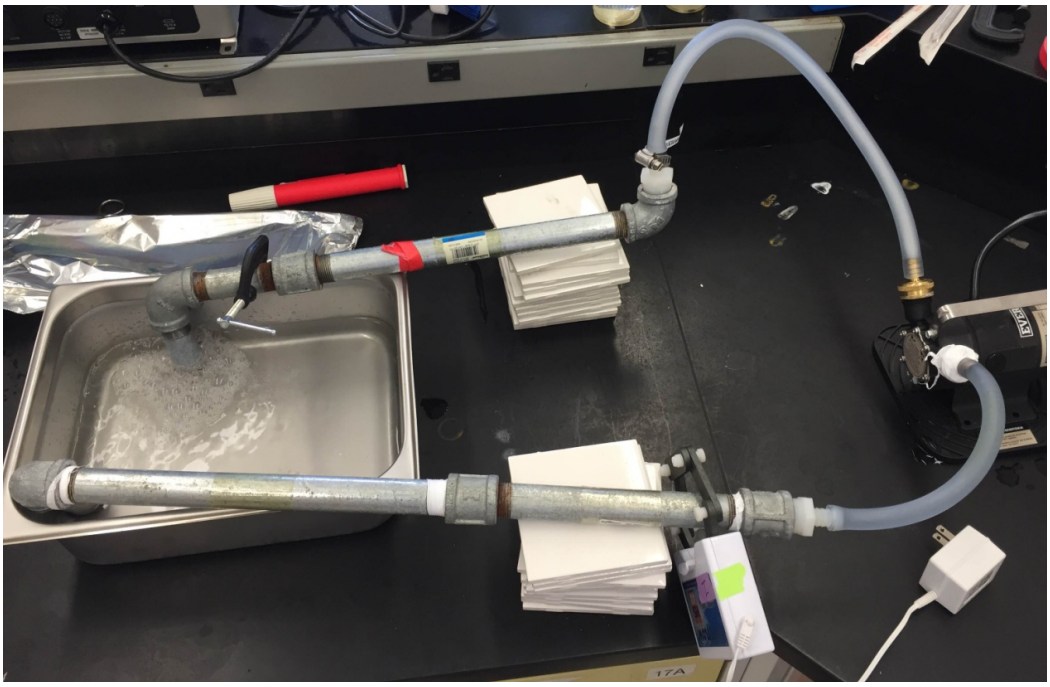

**Figure 2.3. HS48 device in a small volume flow setup containing ¾" galvanized steel piping connected to plastic tubing attached to an Everbilt non-submersible transfer pump.**

***Antimicrobial properties of an alternating current technology on E. coli O157:H7 inoculated into 0.4% NaCl in a large flow system, without metal in the system***

A 78" plastic tube was connected by a brass adapter FGH x Barb to an Everbilt Non-Submersible Transfer Pump on the lead side of the pump. The plastic tubing was connected to the spout of a 25L carboy. On the side leading out of the Everbilt pump, a 58" plastic tubing was secured over the top of the 25L plastic carboy so that the water would flow into the top of the carboy. An HS48 device was placed over the plastic tubing leading into the pump. 20L of a 0.4% NaCl solution in deionized water was added to the carboy for testing.

***Antimicrobial properties of an alternating current technology on S. enteria and E. coli O157:H7 inoculated into 0.4% NaCl in a large flow system, with metal in the system***

A 20 ½" plastic tube was connected by a brass adapter FGH x Barb to an Everbilt Non-Submersible Transfer Pump on the lead side of the pump. The plastic tubing was connected by a ½" x ¾" nylon barb x MIP adapter attached by joiners to an 8" x ¾" galvanized steel pipe. The opposite end of the steel pipe was connected by a joiner to a ½" x ¾" nylon barb x MIP adapter, which was then connected to a 78" plastic tubing. This was all connected to the spout of the 25L carboy water holding tank. On the side leading out of the Everbilt pump, a 58" plastic tubing was secured over the top of the 25L plastic carboy so that the water would flow into the top of the carboy. A model HS48 device was placed over the 8" x ¾" galvanized steel pipe leading into the pump as shown in Figure 2.4. A 20L solution containing 0.4% NaCl in deionized water was added to the carboy for testing.

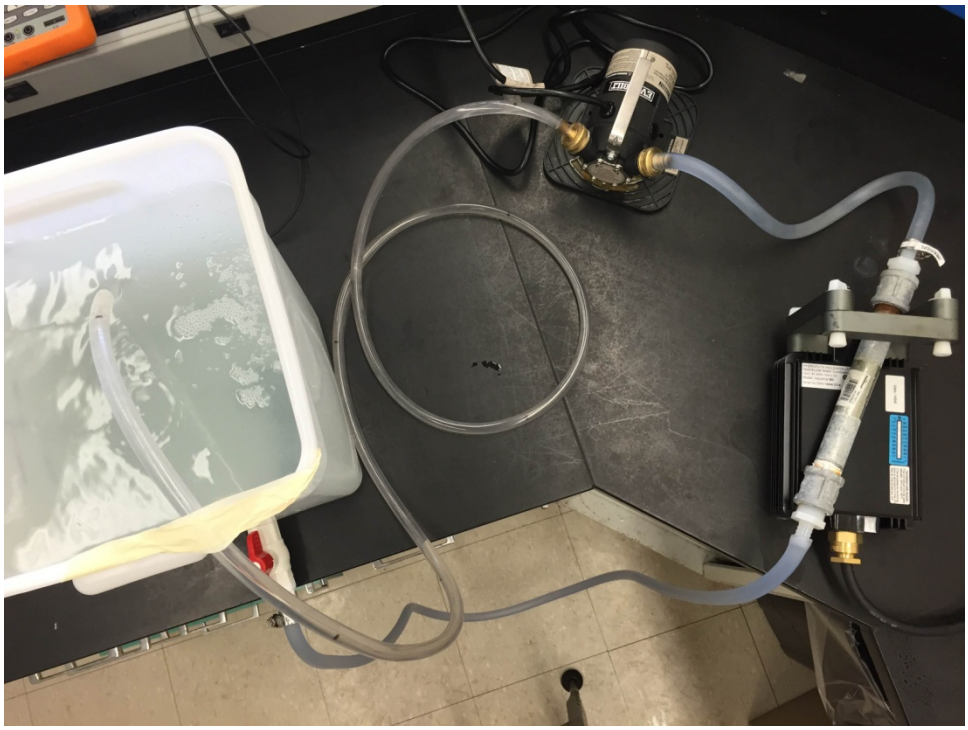

**Figure 2.4. Experimental setup with a 25L carboy water holding tank containing 20L of 0.4% NaCl inoculated with either *S. enteria* or *E. coli* O157:H7. Plastic tubing was connected on either side of a galvanized steel pipe ultimately attaching to an Everbilt Non-Submersible Transfer Pump to circulate water through the piping system. A model 60i device was placed over the steel pipe.**

***Antimicrobial properties of an alternating current technology on Escherichia coli O157:H7 in a 0.3% salt ion solution containing NaCl, $MgCl_2$, $CaCl_2$ and 2.5% Dextrose, without metal in the system***

A flow system was set up as shown in Figure 2.5 using a National Geographic CF30 Aquarium Canister Filter Pump. The pump included a course filter that was kept in the pump as shown in Figure 2.6. A 58" plastic tubing was connected to the canister filter leading away from the pump and secured on the top pouring into a 25L plastic carboy. A plastic hook was placed over top of the 25L carboy and connected to a 96" plastic tubing.

This was then attached to a canister filter pump. A model 60i device was placed over the plastic tubing leading into the pump.

A solution consisting of 20L of deionized water and 36.6g of each salt, NaCl, $MgCl_2$, and $CaCl_2$ as well as 50g of D-Glucose was made in the 25L carboy. Tinfoil was placed over the top of the carboy to avoid splashing and keep foreign particles out of the system.

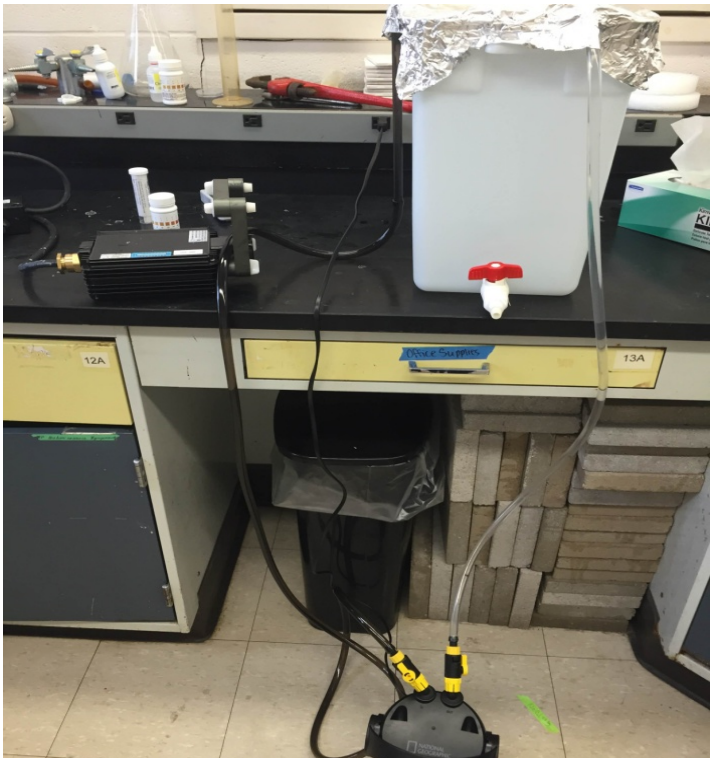


**Figure 2.5. Experimental setup for a large flow system containing a 25L carboy holding tank connected by plastic tubing on either side of a National Geographic CF30 Aquarium Canister Filter Pump with a 60i device secured around the plastic tubing leading into the pump.**

**Figure 2.6. Internal components of a National Geographic CF30 Aquarium Canister Filter Pump containing a prefilter pad used for removal of suspended particles and bulky debris.**

***Antimicrobial properties of an alternating current technology on normal flora in pond water***

A flow system was designed connecting a 58" plastic tubing leading out of a 25L carboy, connected to the output portal of a canister filter pump. A plastic hooked pipe was placed over the side of a 25L carboy, connected to a 96" plastic tubing. This 96" plastic tubing is connected to 3 12" x ¾" galvanized steel pipes, connected by ¾" joiners with a ½" x ¾" nylon barb x MIP adapter on either side connecting the plastic tubing. On the side leading into the intake valve of the canister filter pump is a 20 ½" plastic tube. A model 60i device was placed with the ferrite ring over the steel piping in the middle of the constructed pipe, as shown in Figures 2.7. A second setup was designed with no unit and alternating current device used as the control. Pond water obtained from Ramsey County on 9/10 was measured into a 25L carboy to a final measurement of 20L.

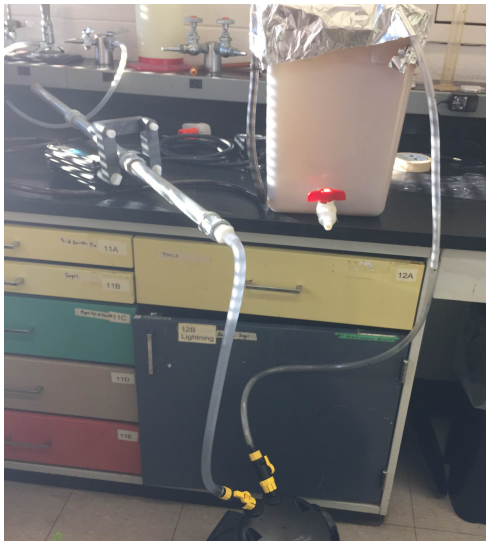

**Figure 2.7. Experimental setup for a large flow system containing a 25L carboy holding tank connected by plastic tubing on the exiting side of a National Geographic CF30 Aquarium Canister Filter Pump and a plastic tubing connected to a longer galvanized piping unit attached to the leading side of the filter pump. A model 60i device was secured around the steel piping leading into the pump.**

## Results and Discussion:

Antimicrobial properties of HS48 ACF on *Salmonella enterica* and *Escherichia coli O157:H7* inoculated into tap water in a static system for 20 minutes

As seen in Figure 2.8 A, *Salmonella enterica* had a 4.07 log cfu/ml reduction in the alternating current frequency (ACF) sample, compared to a 1.79 log cfu/ml reduction in the control sample. *E. coli* O157:H7 had a 1.51 log cfu/ml reduction in the test sample compared to a 2.93 log cfu/ml reduction in the control. *Salmonella* had a lower reduction in the higher inoculum sample compared to the low inoculum, whereas *E. coli* had a similar reduction in the high inoculum as well as the low inoculum test samples - 1.24 log cfu/ml and 1.51 log cfu/ml respectively (Figure 2.8 B).

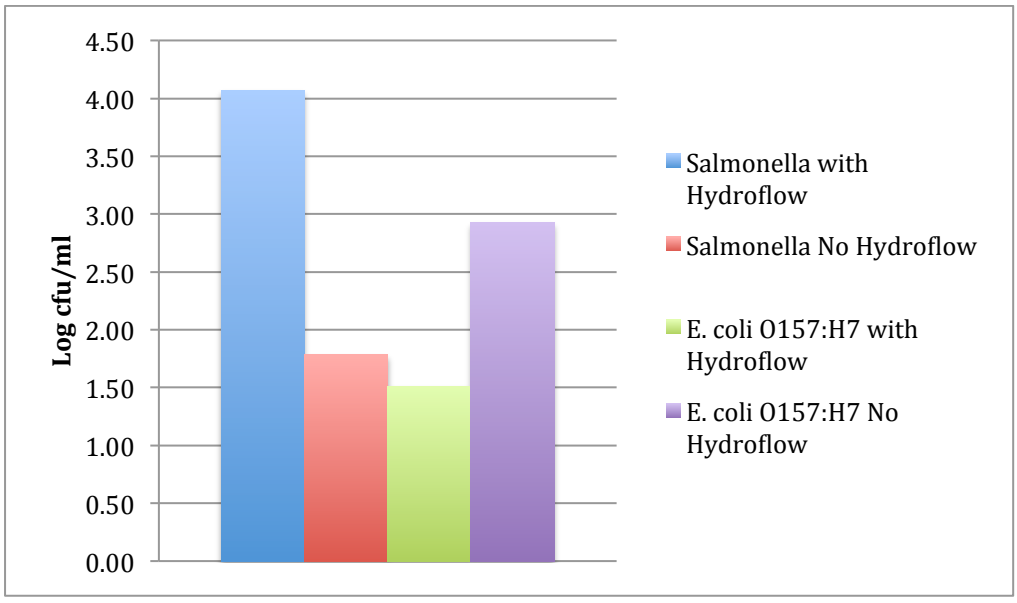


**Figure 2.8 A. Log reduction of *Salmonella* and *E. coli* O157:H7 after 20 minutes exposure to HS48 ACF in tap water with initial inoculum of ~ 4 $\log_{10}$ cfu/ml compared to a control.**

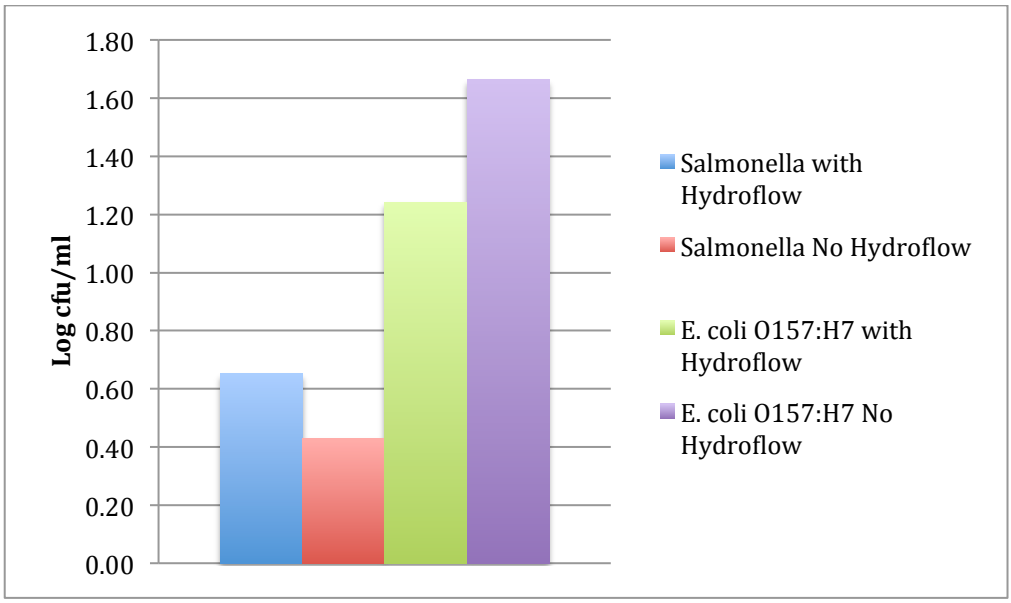


**Figure 2.8 B. Log reduction of *Salmonella* and *E. coli* O157:H7 after 20 min exposure to HS48 ACF in tap water with initial inoculum of ~ 6 $log_{10}$ cfu/ml compared to a control.**

*E. coli* saw a larger reduction in the control compared to the test sample in both the high and low inoculum. Upon further investigation into the raw data from those experiments, samples were not diluted to the full dilution. This caused the controls to report at a greater log reduction than may have been seen if all dilutions had been conducted (Data not shown).

Antimicrobial properties of HS48 ACF on *Listeria monocytogenes, Salmonella enterica* and *Escherichia coli O157:H7* inoculated into tap water in a stirred system for 60 minutes

Cultures of *Listeria monocytogenes, Salmonella enterica* and *Escherichia coli O157:H7* were inoculated into tap water at either ~ 7 $log_{10}$ cf/ml (high concentration) or ~ 4 $log_{10}$

cfu/ml (low concentration). Samples containing a magnetic stir bar were placed on a stir plate and agitated for 60 minutes.

When compared to the control, which demonstrated a complete kill in 60 minutes, there was no observed additional antimicrobial effect due to the HS48 ACF on *Salmonella* or *E. coli* at ~ 4 $\log_{10}$ cfu/ml inoculum. Whereas at the higher ~ 7 $\log_{10}$ cfu/ml inoculum in both *Salmonella* and *E. coli,* there was no observed log reduction in either the HS48 ACF or the control after 60 minutes. This could be due to the higher bioburden in the system.

When comparing static and stirred systems containing *L. monocytogenes*, the static system saw a greater log reduction after 20 min, compared to the stirred system after 30 minutes of exposure to HS48 ACF - 3.49 log cfu/ml and 1.38 log cfu/ml respectively when inoculated with ~ 4 $\log_{10}$ cfu/ml. However, when *L. monocytogenes* was inoculated at ~ 7 $\log_{10}$ cfu/ml, the static system demonstrated only a 0.76 log cfu/ml reduction, compared to 1.8 log cfu/ml in the stirred system.

*Salmonella* and *E. coli* had a lower log reduction after 60 min in a stirred system, compared to a static system at 20 minutes with a high inoculum of ~ 7 $\log_{10}$ cfu/ml and ~6 $\log_{10}$ cfu/ml respectively. Both bacteria also had complete kill in both control and HS48 ACF samples in the low inoculum ~ 4 $\log_{10}$ cfu/ml in the stirred system, compared to the static system where complete kill was not seen in all samples. This can be seen in Figure 2.8A and 2.8B.

Interestingly, the control with no treatment demonstrated antimicrobial activity in tap water. Samples with treatment saw no additional effect when HS48 ACF was added. Upon further investigation it was determined that the tap water contained low levels of antimicrobials. After obtaining the chemical analysis of the water from St Paul municipality, there was reported to be 8ppm of Chloride (as $Cl^{-1}$), 0.09ppm of residual chlorine (as $Cl_2$), and 0.08ppm of Fluoride (as $F^{-1}$) (StPaul.gov). These levels of antimicrobials could have contributed to the log reduction found in both the control and test samples performed with tap water. However, *Salmonella* saw an increased log reduction of 4.07 $\log_{10}$ cfu/ml compared to the control 1.79 $\log_{10}$ cfu/ml. This may be due to the HS48 ACF, combined with the low levels of antimicrobials in the tap water, having a greater effect on bacteria compared to the controls.

Previous research has demonstrated that electric current, in combination with antimicrobials, has a greater effect on bacteria compared to the antimicrobial alone (Wellman et al., 1996, Costerton et al., 1994, Giladi et al., 2008). This may be why there is a greater log reduction in the test samples when compared to the control in samples containing tap water. However, the agitation from the stirred system may have allowed low inoculum test samples to come into contact with the low level antimicrobials and prevent bacteria from settling out of solution, resulting in a high log reduction.

Antimicrobial properties of HS48 ACF on *S. enteria*, and *E. coli* O157:H7 inoculated into deionized water in a static system for 60 minutes

To control for the presence of antimicrobials in tap water, cultures of *Listeria monocytogenes*, *Salmonella enterica* and *E. coli* O157:H7 were inoculated in deionized

water to determine the effect of the alternating current treatment without the presence of additional biocides or ions. Results demonstrated over the 60 minutes that all three bacterial cultures remained stable compared to the control. With an initial inoculum of approximately ~ 4 log cfu/ml in both control and alternating current treated solutions, there was no reduction in bacterial populations (Data not shown).

After further discussion into the mechanism of the alternating current technology, it was determined that the mechanism required ions to be present in the system to demonstrate biocidal activity.

Effect of alternating current technology on water physiochemistry with the addition of 0.9% NaCl

A flow through system was designed to reproduce field conditions on a lab bench scale. Addition of 2L of 0.9% NaCl was used to increase ions to aid in the efficacy of the alternating current treatment. Physiochemical analysis was performed to determine the effect of the ACF on the change in pH, ORP, temperature, and production of free available chlorine (FAC). The change in pH, as seen in Table 2.1, could be attributed to the increase in FAC detected over time. The ORP saw an increase of approximately 50mV over the 4 hours, which may also be due to the increased FAC as well as to the introduction of oxygen into the system. The temperature increase can be attributed to the small volume of liquid used in the system as well as the pump used, which produced a large amount of heat while circulating over the 4 hours of testing. After initial testing, it was decided that a larger apparatus would be used to control for the heat produced by the

flow through system, in order to investigate the efficacy of ACF without the addition of heat.

**Table 2.1. Measurement of pH, ORP, temperature and free available chlorine over 4 hours in a 0.9% Saline solution exposed to a HS48 ACF in a small flow system.**

| Time | pH | ORP (mV) | Temperature (°C) | FAC |
|---|---|---|---|---|
| 0 hours | 8.03 | 235.33 | 20.40 | 0-10ppm |
| 1 Hour | 7.20 | 254.67 | 31.60 | 25ppm |
| 3 Hours | 7.50 | 258.33 | 35.17 | 25ppm |
| 4 Hours | 7.50 | 271.00 | 34.80 | 25ppm |

Antimicrobial properties of an alternating current technology on *Salmonella enterica* and *Escherichia coli O157:H7* inoculated into 0.4% NaCl in a large flow system

***Escherichia coli O157:H7 in 0.4% NaCl exposed to a model HS48 over plastic tubing***

A large flow apparatus was designed to reproduce field conditions on a lab bench scale. Addition of 0.4% NaCl was used in order to determine the impact on the efficacy of the ACF treatment. The holding tank, filled with 20L of 0.4% NaCl solution, was inoculated with 4.25 log cfu/ml of *E. coli* O157:H7. Over the 4 h of testing, there was no change in log of bacteria. ORP did increase after the initiation of the flow thru system, but remained constant over the next 3 hours, as seen in Table 2.2. The larger system also allowed temperature to remain stable over the 4 hours of testing.

To address the low log reduction, a new experimental apparatus included a larger alternating current unit to increase signal strength. Metal conduction in the form of a

stainless steel pipe was also added to the system to help propagate the signal through the system.

**Table 2.2. Measurement of pH, ORP and temperature over 4 hours. Log change in *E. coli* O157:H7 counts from initial inoculum of 4.25 log cfu/ml over 4 hours in a 0.4% NaCl solution exposed to a HS48 ACF in a large flow system.**

| Sample | Time | pH | ORP (mV) | Temperature (°C) | Δ $Log_{10}$ |
|---|---|---|---|---|---|
| **Alternating Current** | 0 hours | 6.3 | 262 | 23.1 | |
| | 1 Hour | 5.2 | 930 | 23.9 | 0.05 |
| | 2 Hours | 5.2 | 930 | 22.9 | 0.06 |
| | 3 Hours | 5.4 | 893 | 24.5 | 0.00 |
| | 4 Hours | 5.5 | 873 | 28 | -0.01 |

***Escherichia coli O157:H7 in 0.4% NaCl exposed to a model 60i over a metal pipe:***

Results demonstrated that, when using a 60i ACF combined with metal, there was a 1-log cfu/ml decrease in *E. coli* population over 4 hours when compared to the HS48 ACF over the same timeframe (see Table 2.3). ORP initially increased once the flow system was initiated, but stabilized over the 4 hours, as seen in the previous experiment. This can be due to the increase in oxygen after initiation of the flow system. After these preliminary experiments showed promising results, controls were added and the testing was taken out to extended time points to detect the continued trend.

**Table 2.3. Measurement of pH, ORP and temperature over 4 hours. Log change in *E. coli* O157:H7 counts from initial inoculum of 4.29 log cfu/ml over 4 hours in a 0.4% NaCl solution exposed to a 60i ACF in a large flow system containing metal.**

| Sample | Time | pH | ORP (mV) | Temperature (°C) | Δ $Log_{10}$ |
|---|---|---|---|---|---|
| **Alternating Current** | 0 hours | 5.9 | 423 | 23.1 | |
| | 1 Hour | 5.5 | 829 | 23.7 | 0.04 |
| | 2 Hours | 5.8 | 820 | 25.7 | -0.06 |
| | 3 Hours | 6.1 | 813 | 26.8 | -0.16 |
| | 4 Hours | 6.3 | 807 | 27.3 | -1.22 |

***Escherichia coli O157:H7 and Salmonella enterica in 0.4% NaCl exposed to a model 60i over a metal pipe:***

After the preliminary experiment showed a steady decline in population over the 4 hours of testing, the same experiment was run with a control to determine the difference in exposure to 60i ACF. The test sample with 60i ACF was inoculated with 4.32 log cfu/ml of *E. coli*. The control with no current had a starting inoculum of 4.29 log cfu/ml. After 5.5 hours, both control and test samples containing *E. coli* saw complete reduction, as seen in Table 2.4.

When the same experiment was performed with *S. enterica*, test samples exposed to 60i ACF decreased at a greater rate compared to the control over 4 hours. At 3 hours, 60i ACF treated samples had a 1.33 log cfu/ml reduction compared to a 0.81 log cfu/ml reduction in the control. After 4 hours of sampling, the alternating current demonstrated a 2.23 log cfu/ml reduction compared to a 1.89 log cfu/ml reduction in the control, as shown in Table 2.4.

When *E. coli* and *Salmonella* were compared under the same conditions in a 0.4% NaCl solution exposed to a 60i ACF over metal piping, both bacteria had a greater kill in the 60i ACF samples compared to the control. Samples containing *Salmonella,* greater log reduction was seen at the 3 and 4 hour time points, whereas *E. coli* had a greater reduction at the 4 hour time point when compared to the control, as shown in Table 2.4. The *E. coli* control showed complete kill at 5 hours, whereas the 60i ACF sample did not show complete kill until 5.5 hours.

Calculations were done to determine the limit of detection. The liquid was sampled by pipetting 1ml of liquid out of 20 liters of test solution, making the limit of detection 1log. Therefore, we cannot say that we were able to see complete kill after 5.5 hours. However, the low buffer concentration may have caused stress on the cells, resulting in the low recovery. A new solution was developed, comprised of salts found in field conditions as well as those utilized in similar electric current studies (Davis et al., 1989, 1991, Zita and Hermansson 1994, Caubet et al., 2004).

Investigation was done to determine other factors that could be contributing to the drop in bacterial counts in both test and control samples. Shear rate can attribute to the dramatic decrease of bacterial counts due to the rate of flow from the pump and low viscosity of the solution. Using the following equation we can calculate the velocity of the system at 4.82m/s.

$$V=q_v/\pi R^2$$

At this rate there is an increased amount of pressure put on the cell resulting in cell death. Thus this experimental design appears to have artificially augmented the efficacy of the ACF.

**Table 2.4. Log change in bacteria over time in a system containing *E. coli* O157:H7 with an initial inoculum of 4.32 log cfu/ml in the test sample and 4.29 log cfu/ml in the control. Compared to *Salmonella enterica* with an initial inoculum of 4.25 log cfu/ml in the test sample and 4.16 log cfu/ml in the control in a 0.4% NaCl solution exposed to a 60i in a large flow system containing metal.**

| Sample | Time | Δ $Log_{10}$ *E. coli* | Time | Δ $Log_{10}$ *S. enterica* |
|---|---|---|---|---|
| **Alternating Current** | 0 hours | | 0 Hour | |
| | 1 Hour | 0.05 | 1 Hour | -0.32 |
| | 2 Hours | -0.07 | 2 Hours | -0.54 |
| | 3 Hours | -0.37 | 3 Hours | -1.33 |
| | 4 Hours | -2.24 | 4 Hours | -2.23 |
| | 5 Hours | -3.42 | - | - |
| | 5.5 Hours | -4.32 | - | - |
| **Control** | 0 hours | | 0 Hour | |
| | 1 Hour | -0.07 | 1 Hour | - |
| | 2 Hours | 0.02 | 2 Hours | -0.21 |
| | 3 Hours | -0.40 | 3 Hours | -0.81 |
| | 4 Hours | -2.01 | 4 Hours | -1.89 |
| | 5 Hours | -4.29 | - | - |
| | 5.5 Hours | -4.29 | - | - |

Antimicrobial properties of an alternating current technology on *E. coli* O157:H7 inoculated into a 0.3% salt ion solution containing NaCl, $MgCl_2$, $CaCl_2$ and 2.5% Dextrose in a large flow system

After the results from the previous experiment showed log reduction in both the test and control samples, a solution comprised of increased salt ions plus dextrose was designed to introduce a more field based application as well as determine the effect of the alternating current with the addition of ions on the bacteria in solution.

There was a similar increase in *E. coli* concentration in the test and control samples. The sample exposed to alternating current showed a 0.74 log increase compared to the initial time 0. However, there was an initial drop at the 1-hour time point. This may be attributed to insufficient circulating of the bacteria culture before initial sampling at time 0. Therefore, if the log change is calculated based on the 1-hour time point then the log increase is 0.98 log cfu/ml in the test sample compared to a 0.93 log cfu/ml increase in the control sample. Therefore, we cannot conclude significant difference within the 24 hours, as seen in Table 2.5.

When comparing this to the data presented in Table 2.4, which only contained NaCl in the system, there was an increase in bacterial population and no observed difference between the treated and control samples after 5 hours. This trend continued over the 24 hours of testing.

Physiochemistry of both systems changed over the course of testing. The pH in both the test and control samples dropped from ~ 6.0 to ~3.8 over 24 hours. ORP readings increased to ~ 50mV to ~ 190 mV in both the test and control samples, as can be seen in Table 2.5. This can be due to the increased oxygen added to the system over a 24 hour testing period.

The new method was used as described above, adding increased ions along with dextrose to the system. As described in Kirson et al., (2004), dividing cells had a greater

susceptibility to alternating electric fields. Therefore, we would expect to see the alternating current having a superior effect on bacterial cells during growth phase. However, this was not observed. An increased bacterial concentration was used to determine if a high bioburden would produce similar results to a low bioburden. The results seen in this experiment are possibly due to the lack of metal in the system to aid in propagating the signal, as well as longer time points to achieve log reduction. Case studies conducted by the contracted company have shown log reduction after days as well as hours.

Velocity of the system may have contributed to the difference in log recovery between this and previous experiments. Previous experimental design utilized a larger pump (1362.8L/hr), while the current setup employed a smaller pump with a throughput of 499.7L/hr. The velocities of the systems were 4.82 m/s and 1.77 m/s respectively. The addition of glucose to the system would also decrease velocity of the system by increasing viscosity of the fluid. This would put less pressure on the bacterial cells compared to the previous study, making it a non-contributing factor to the log change (Table 2.5), compared to the log decrease effected by the previous design, as seen in Table 2.4.

**Table 2.5. Measurement of pH, ORP and temperature over 24 hours. Log change in *E. coli* O157:H7 counts from initial inoculum of 6.56 log cfu/ml in the test sample and 6.27 log cfu/ml in the control over 24 hours in a 0.3% salt ion solution containing NaCl, $MgCl_2$, $CaCl_2$ and 2.5% Dextrose exposed to a 60i ACF over a plastic tubing in a large flow system. Adjusted Δ $Log_{10}$ calculated to the 1-hour time point with a concentration of 6.32 log cfu/ml in the test sample and 6.31 log cfu/ml in the control.**

| Sample | Time | pH | ORP (mV) | Temperature (°C) | Δ $Log_{10}$ | Adjusted Δ $Log_{10}$ |
|---|---|---|---|---|---|---|
| **Alternating Current** | 0 hours | 6.0 | 63.5 | 23.3 | | |
| | 1 Hour | 5.9 | 69.2 | 23.4 | -0.24 | |
| | 2 Hours | 5.8 | 70.9 | 23.3 | -0.17 | 0.07 |
| | 3 Hours | 5.7 | 78.0 | 23.1 | 0.12 | 0.36 |
| | 4 Hours | 5.4 | 87.8 | 23.0 | 0.12 | 0.37 |
| | 5 Hours | - | - | - | 0.39 | 0.63 |
| | 6 Hours | 5.1 | 113.1 | 22.7 | 0.45 | 0.69 |
| | 7 Hours | 5.0 | 119.5 | 22.6 | 0.41 | 0.65 |
| | 8 Hours | 4.8 | 127.0 | 22.6 | 0.56 | 0.80 |
| | 12 Hours | - | - | - | 0.67 | 0.91 |
| | 24 Hours | 3.7 | 191.6 | 22.8 | 0.74 | 0.98 |
| **Control** | 0 hours | 6.1 | 53.0 | 23.1 | | |
| | 1 Hour | 6.1 | 55.1 | 23.1 | 0.04 | |
| | 2 Hours | 6.0 | 62.3 | 23.1 | 0.22 | 0.19 |
| | 3 Hours | 5.9 | 67.0 | 23.2 | 0.37 | 0.33 |
| | 4 Hours | 5.7 | 75.0 | 23.2 | 0.26 | 0.23 |
| | 5 Hours | - | - | - | 0.64 | 0.60 |
| | 6 Hours | 5.2 | 99.2 | 23.1 | 0.77 | 0.73 |
| | 7 Hours | 5.1 | 115.1 | 23.0 | 0.82 | 0.78 |
| | 8 Hours | 4.8 | 128.1 | 23.0 | 0.82 | 0.78 |
| | 12 Hours | - | - | - | 0.96 | 0.92 |
| | 24 Hours | 3.8 | 190.9 | 23.3 | 0.96 | 0.93 |

Antimicrobial properties of an alternating current technology on normal flora in pond water

After the addition of increased salt ions plus dextrose saw no difference between ACF and control samples, pond water was used to further mimic field conditions. This alternating current technology has been used to aid in the reduction of unwanted algae and other contaminating bacteria from ponds and man-made lagoons. Normal aerobic flora was tracked over 49.5 hours. After 49.5 hours of exposure to 60i ACF, there was no difference in log change when compared to the control. The ACF sample saw an increase of 2.35 log cfu/ml from an initial sampling of 3.94 log cfu/ml. Control samples increased 1.98 log cfu/ml from an initial sampling of 4.04 log cfu/ml, as seen in Table 2.6.

Testing was done by sampling aerobic bacteria, therefore results for other bacteria - especially anaerobic and unculturable bacteria - were not captured in this experiment, even though they may have been affected by the ACF. In field testing performed by this company, sampling is done using SaniCheck® agar dip slides. The use of this sampling technique on the surface of the water would not detect those bacteria that may flocculate and settle to the bottom of the system.

However, if flocculation of particles is occurring and settling out of solution, this may lead to greater kill while under anaerobic conditions. It has been suggested that there are large differences in the effect of magnetic fields on bacteria, depending on the presence of dissolved oxygen. In Kohno et al., (2000), it was shown that magnetic fields had more

effect under anaerobic conditions. Therefore, in a field situation where anaerobic conditions can be achieved, a greater difference may be seen compared to a lab system.

Upon further investigation, it was discovered that the ACF signal is able to travel farther than initially thought. Although test and control samples were kept at opposite sides of the lab, it is possible that signal from the ACF device was able to travel and affect the control system. This could explain the similar results seen across bacterial counts as well as physiochemistry between experiments.

**Table 2.6. Measurement of pH, ORP, temperature, and conductivity over 24 hours. Log change in Aerobic normal flora counts from initial inoculum of 3.94log cfu/ml in the test sample and 4.04 log cfu/ml in the control over 49.5 hours in pond water 60i ACF in a large flow system containing metal.**

| Sample | Time | pH | ORP (mV) | Temperature (°C) | Conductivity µS/cm | Δ $Log_{10}$ |
|---|---|---|---|---|---|---|
| **Alternating Current** | 0 Hours | 7.03 | -1.7 | 21.6 | 870.0 | 3.94 |
| | 1 Hour | 7.06 | -3.9 | 22.0 | 810.0 | 0.77 |
| | 2 Hours | 7.08 | -6.3 | 22.2 | 840.0 | 0.94 |
| | 3 Hours | 7.12 | -7.8 | 22.5 | 860.0 | 0.96 |
| | 4 Hours | 7.99 | -10.9 | 22.7 | 850.0 | 1.17 |
| | 5 Hours | 7.23 | -13.4 | 22.9 | 850.0 | 1.18 |
| | 6 Hours | 7.28 | -16.7 | 23.0 | 850.0 | 1.30 |
| | 7 Hours | 7.32 | -18.9 | 23.1 | 860.0 | 1.33 |
| | 8 Hours | 7.37 | -21.8 | 23.2 | 860.0 | 1.80 |
| | 24 Hours | 7.73 | -42.9 | 22.4 | - | 2.28 |
| | 31 Hours | 7.82 | -48.2 | 22.7 | - | 2.64 |
| | 49.5 Hours | 8.00 | -58.9 | 22.8 | 830.0 | 2.35 |
| **Control** | 0 Hours | 7.03 | -1.7 | 21.6 | 870.0 | 4.04 |
| | 1 Hour | 6.97 | 1.9 | 22.0 | 800.0 | 1.54 |
| | 2 Hours | 7.01 | -0.4 | 22.2 | 820.0 | 1.58 |
| | 3 Hours | 7.02 | -1.2 | 22.5 | 830.0 | 1.89 |
| | 4 Hours | 7.04 | -2.4 | 22.6 | 820.0 | 2.25 |
| | 5 Hours | 7.06 | -3.3 | 22.9 | 830.0 | 2.22 |
| | 6 Hours | - | - | - | 830.0 | 2.24 |
| | 7 Hours | 7.21 | -12.3 | 23.1 | 810.0 | 2.14 |
| | 8 Hours | 7.17 | -9.8 | 23.2 | 860.0 | 2.29 |
| | 24 Hours | 7.46 | -26.8 | 22.6 | - | 2.19 |
| | 31 Hours | 7.64 | -37.3 | 22.8 | - | 2.68 |
| | 49.5 Hours | 7.67 | -39.4 | 22.7 | 850.0 | 1.98 |

We were unable to produce an operative model design that would demonstrate the effectiveness of ACF in a small or large scale flow system. This may be due in part to a hypothesized magnetic loop that is created when this alternating current technology is placed in a small system. This inability for the signal to spread throughout the system may contribute to the insignificant differences observed between test and control samples.

Due to the inability to design an operational testing apparatus that demonstrated the effectiveness of the ACF, a new method approach was taken. Based on the initial study using tap water in a static system that showed an increased efficacy of the ACF, along with previous research investigating the combined effect of electric current with antimicrobials (Costerton et al., 1994, Lopez-Galvez et al., 2012, Giladi et al., 2008, Mirzaii et al., 2015), a new method, designed to study the effect of the ACF on bacteria when exposed to varying levels of antimicrobial is warranted.

# Chapter 3:
# Enhanced Biocidal Effect of Alternating Current in Combination with Antimicrobials in a Static System

## Introduction:

As the global population attempts to move into a more "eco-friendly" world and reduce the amount of chemicals used to clean the environment as well as our food, we must investigate ways that allow for the reduction of chemical usage while still producing the same results. One method that has been investigated is the combination of electric current with a reduced concentration of antimicrobial substances. There has been evidence that electric current facilitates the efficacy of an antimicrobial (Costerton *et al*., 1994, López-Gálvez *et al*., 2012, Giladi *et al*., 2008, Mirzaii *et al*., 2015). Studies have been reported using both planktonic bacteria as well as bacteria in biofilms (Costerton *et al*., 1994, Liu *et al*., 1997, Jeong *et al*., 2009, Mirzaii *et al*., 2015).

Previous studies have shown that the core purpose of sanitizers in a process scenario is to maintain microbial safety of process water while avoiding cross contamination (López-Gálvez *et al*., 2010a). However, when using chlorine-based sanitizers, chlorine can react with organic matter present in the water and subsequently forms potentially harmful chlorinated by-products in process water (López-Gálvez *et al*., 2010b). Therefore it is important to develop new technologies that enable the reduction of the level of chlorine

required for disinfection, as well as reduce the level of organic matter in the process water (López-Gálvez *et al*., 2012).

Previous studies have utilized varying solutions for bacterial suspension when investigating the efficacy of alternating current. Buffered solutions have been utilized to test adhesion of bacteria to surfaces (Seok *et al*., 2008, Shim *et al*., 2011). Growth media including Mueller-Hinton agar (Maadi *et al*., 2010), LB broth (Giladi *et al*., 2008), agar (Fotj *et al*., 2009) and Tryptic Soy Broth (Inhan-Garip *et al*., 2011, Mirzaii *et al*., 2015) as well as salt solutions such as M56 (Caubet *et al*., 2004), potassium and calcium chloride (Zita and Hermanson 1994), and synthetic urine (Davis *et al*., 1989, 1991) have been used to determine the effect of electric fields on bacteria.

Time points for exposure to ACF were chosen based upon previously described studies. Inhan-Garip *et al*., (2011) described *E. coli's* adaptive response to extremely low frequency electromagnetic fields of 50Hz after 6 hours. It was suggested that the magnetic field effect is maximized in the first hours of exposure, and then decreases over time, implying that there is an adaptive response by the cells. Previous studies have shown a decreased ability for bacteria to form colonies with the increase of magnetic field intensity as well as exposure time up to 120 minutes (Strasak *et al*., 2002; Fojt *et al*., 2004; El-Sayed *et al*., 2006). In this experiment, several time points were chosen to incorporate both what has been described in the literature as well as what has been observed in the field. Longer time points were chosen to determine exposure effects as

shown in Giladi *et al*., (2008), where 48 hours of exposure to 150 kHz alternating current was shown to be bacteriostatic.

In this study, we utilized previously described solutions while incorporating field applicable conditions. Different salt and buffered solutions were investigated to determine the effect of the suspension solution combined with the alternating current frequency on the susceptibility to subsequent exposure to antimicrobials. Growth media was not used, as the time points chosen would have led to an undesired population increase.

This study investigated the effect of the alternating current frequency (ACF) on the susceptibility of *Escherichia coli* 0157:H7 bacteria to Sodium Hypochlorite. It was hypothesized that bacteria, when exposed to antimicrobials and grown in a minimal media, would recover slower and grow at a reduced rate after exposure to ACF compared to bacteria not subjected to electric current. The method chosen to investigate this hypothesis was a Minimal Inhibitory Concentration (MIC) Plating Procedure. This method was adapted from the CLSI Method for Dilution Antimicrobial Susceptibility tests for Bacteria that Grow Aerobically.

## Minimal Inhibitory Concentration Materials and Methods:

Culture conditions:

Bacterial strain *Escherichia coli* O157:H7, obtained from the University of Minnesota culture collections of environmental isolates, was grown in 10ml of Bacto™ Tryptic Soy Broth (TSB) Soybean-Casein Digest Medium made by BD. Bacterial cultures were kept in freezer conditions in glycerol. Cultures were serially transferred twice from freezer stock and grown overnight ~ 18 hours in TSB at 37°C. Final concentration of the overnight culture was ~$10^9$ cfu/ml. This was used as the working stock culture for sample inoculation. Samples were inoculated with 2ml from working stock cultures to achieve ~$10^7$cfu/ml final concentration.

Microbial analysis:

For the purposes of bacterial growth, Bacto™ Tryptic Soy Broth Soybean-Casein Digest Medium made by BD for bacteria cultures was used. Aerobic Plate Count (APC) 3M® Petrifilm was used for bacterial growth.

For sample enumeration, 1ml of sample was pipetted out of the test sample and serially diluted twice 1:10 into standard sterile saline dilution broth (0.9% saline). Samples were diluted to a countable range and plated on 3M® Aerobic Plate Count (APC) Petrifilm. Plates are incubated at 37°C for 48 hours then read for colony formation.

Alternating current model units:

Three different model units - model HS48 units: serial number 3105420112, serial number GMA-264785 01/11, model 60i serial number GMA-145403714, and a model 100i serial number GMA–14703.

Sample preparation:

Figure 3.1 illustrates the variable conditions tested in this chapter to study the differences in bacterial growth patterns when subjected to ACF under different suspension conditions and subsequently grown in minimal media containing different concentrations of antimicrobial.

| Bacterial Strain | Liquid Solution | Container Type | Hydroflow Model | Sample Time Points | Antimicrobial |
|---|---|---|---|---|---|
| *Escherichia coli* O157:H7 | 0.9% Saline | Glass Erlenmeyer Flask | HS48 60i | 6, and 24 Hours | Sodium Hypochlorite (Bleach) |
| *Escherichia coli* O157:H7 | BPW | Glass Erlenmeyer Flask | HS48 60i | 6, 24, and 48 Hours | Sodium Hypochlorite (Bleach) |
| *Escherichia coli* O157:H7 | BPW | Stainless Steel Container | 100i | 6, 24, and 48 Hours | Sodium Hypochlorite (Bleach) |
| *Escherichia coli* O157:H7 | 0.3% ion salt solution<br>0.75g KCl<br>0.75g NaCl<br>0.75g MgCl<br>0.75g CaCl | Stainless Steel Container | 100i | 6, 24, and 48 Hours | Sodium Hypochlorite (Bleach) |

**Figure 3.1. Experiment schematic for MIC testing of HS48, 60i and 100i ACF using *Escherichia coli* O157:H7 bacteria in different liquid mediums and container types.**

***Effect of HS48 ACF on Escherichia coli O157:H7 in 0.9% saline or BPW in a static glass system exposed to Sodium Hypochlorite:***

For this test, 200ml of either 0.9% saline or BBL™ Buffered Peptone Water (BPW) containing 1.0% Peptone, 0.5% Sodium Chloride, 0.35% Disodium Phosphate, and 0.15% Monopotassium Phosphate, purchased from BD, was measured into a sterile

250ml glass Erlenmeyer flask. A HS48 ACF unit was placed over the sample as shown in Figure 3.2. Control samples contained no unit and were kept in a separate room to control for signal transfer from the HS48 ACF test samples.

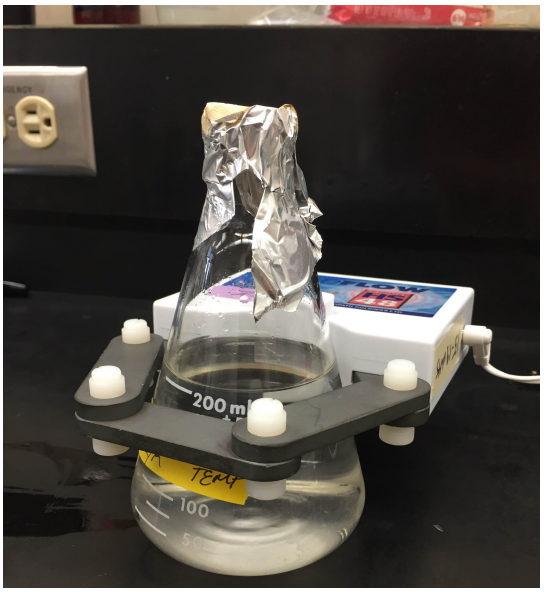


**Figure 3.2. Experimental setup testing model HS48 over a 250ml glass Erlenmeyer flask containing 200ml of either a 0.9% saline or BPW solution.**

***Effect of 60i ACF on Escherichia coli O157:H7 in 0.9% saline or BPW in a static glass system exposed to Bleach:***

For this test, 200ml of either 0.9% saline or BBL™ Buffered Peptone Water (BPW), purchased from BD, was measured into a sterile 250ml glass Erlenmeyer flask. A 60i ACF unit was placed over the sample as shown in Figure 3.3. Control samples contained no unit and were kept in a separate room to control for signal transfer from the 60i ACF test samples.

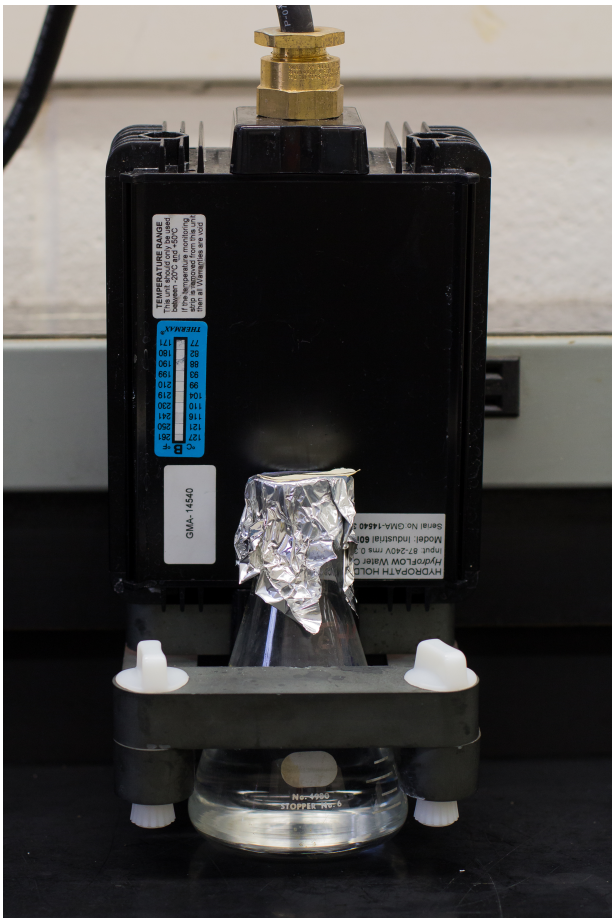


**Figure 3.3. Experimental setup testing model 60i over a 250ml glass Erlenmeyer flask containing 200ml of either a 0.9% saline or BPW solution.**

***Effect of 100i ACF on Escherichia coli O157:H7 in BPW or a 0.3% ion solution in a static stainless steel system exposed to sodium hypochlorite (Bleach):***

For this test, 200ml of either BBL™ Buffered Peptone Water (BPW) or a salt solution consisting of 0.75g measured out of each salt, KCl, NaCl, $MgCl_2$, and $CaCl_2$, was measured into a sterile 250ml glass Erlenmeyer flask. The sample was placed in the center of the ferrite ring of a 100i ACF unit as shown in Figure 3.4. Control samples contained no test unit and were kept in a separate room to control for signal transfer from the 100i ACF test samples.

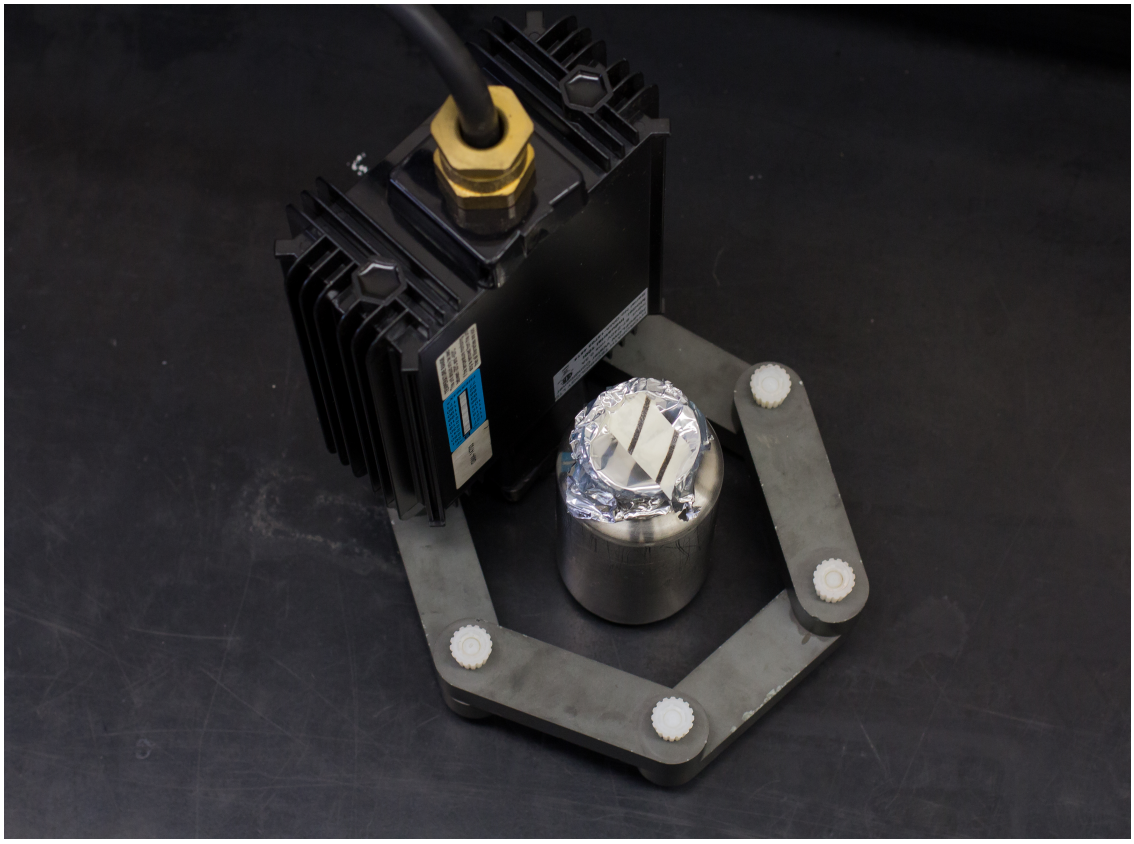

**Figure 3.4. Experimental setup testing model 100i over a stainless steel container containing 200ml of either a BPW or 0.3% ion salt solution.**

***Preparation of 96 well microtiter MIC plate 8-way:***

The total volume of each well should be 100µl. 50µl of sterile Mueller Hinton Broth (MHB) was pipetted into each well that will contain antimicrobial dilutions. Each antimicrobial was diluted into MHB to twice the desired final well concentration of the antimicrobial. 100µl of the mixture of antimicrobial and MHB was pipetted into column 1 of a Costar EIA/RIA Flat bottom, with low evaporation lid Polystyrene 96-well microtiter plates. 50µl was pipetted out of Column 1 and mixed with the MHB in

Column 2 by pipetting up and down 5-10 times. Pipette tips were discarded. New tips are used to pipette 50µl from Column 2 to Column 3 and mixed 5-10 times again. Pipette tips were discarded, and new tips were used. This is repeated to Column 10, removing 50µl from the final column and discarding it. Well 11A did not contain any antimicrobial, as it was the negative control. Column 12 also did not contain antimicrobial and was used as the positive growth control.

Microtiter 96 well plates were prepared before sampling at each time point to ensure the lowest possible time between the termination of bacterial exposure to the alternating current and exposure to the antimicrobial in the well. The first 1:10 dilution from the test samples, as described above, was poured into a sterile petri dish. 50µl of this sample was pipetted into each well containing antimicrobial dilutions. Each sample was plated in duplicate rows on the 96 well plate. Each well, except the negative control, contained ~5 x $10^4$ cfu/ml. The last column contained no antimicrobial, and was used as the positive growth control. 50µl of sterile 0.9% saline was added to the negative control well (11A). The final plate scheme can be seen in Figure 3.5.

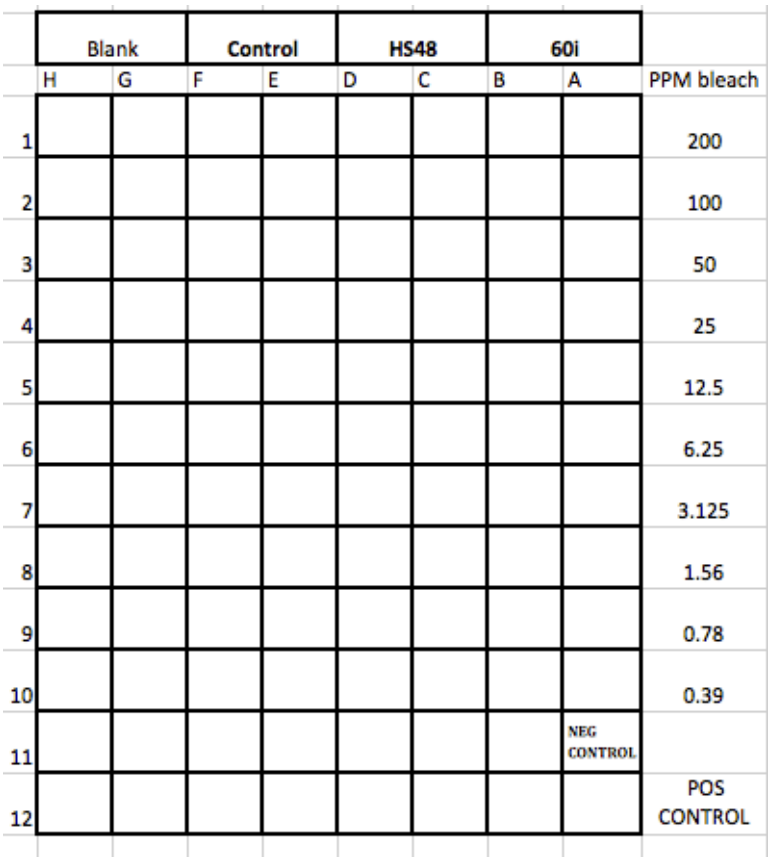


**Figure 3.5. 96 well microtiter plating schematic for MIC testing 8-way, with model HS48 and 60i in Bleach compared to a control.**

***Preparation of 96 well microtiter MIC plate 12-way:***

The total volume of each well should be 100µl. 50µl of sterile Mueller Hinton Broth (MHB) was pipetted into each well that will contain antimicrobial dilutions. Each antimicrobial was diluted into MHB to twice the desired final well concentration of the antimicrobial. 100µl of the mixture of antimicrobial and MHB was pipetted into row A of a Costar EIA/RIA Flat bottom, with low evaporation lid Polystyrene 96-well microtiter plates. 50µl was pipetted out of row A and mixed with the MHB in row B by pipetting up and down 5-10 times. Pipette tips were discarded. New tips are used to pipette 50ul from row B to row C and mixed 5-10 times again. Pipette tips were discarded. This is repeated to row F, removing 50µl from the final column and discarding it. The negative control well 12G did not contain antimicrobial. Row H also did not contain antimicrobial; and was used as the growth (positive) control.

Microtiter 96 well plates were prepared before sampling at each time point to ensure the lowest possible time between the termination of bacterial exposure to the alternating current and exposure to the antimicrobial in the well. The first 1:10 dilution from the test samples, as described above, was poured into a sterile petri dish. 50µl of this sample was pipetted into each well containing antimicrobial dilutions. Each sample was plated in duplicate rows on the 96 well plate. Each well, except the negative control, contained ~5 x $10^4$ cfu/ml. The last row should contain no antimicrobial, and was used as the positive growth control. 50µl of sterile 0.9% saline was added to the negative control well (12G). The final plate scheme can be seen in Figure 3.6 A and B.

| | 60i | | | HS48 | | | Control | | | Blank | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | PPM bleach |
| A | | | | | | | | | | | | | 200 |
| B | | | | | | | | | | | | | 100 |
| C | | | | | | | | | | | | | 50 |
| D | | | | | | | | | | | | | 25 |
| E | | | | | | | | | | | | | 12.5 |
| F | | | | | | | | | | | | | 6.25 |
| G | | | | | | | | | | NEG CONTROL | | | |
| H | | | | | | | | | | | | | POS CONTROL |

**Figure 3.6 A. 96 well microtiter plating schematic for MIC testing 12-way, with model HS48 and 60i in Bleach compared to a control.**

| | | 100i | | | Control | | | 100i | | | Control | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PPM Bleach | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 200 | A | | | | | | | | | | | | |
| 100 | B | | | | | | | | | | | | |
| 50 | C | | | | | | | | | | | | |
| 25 | D | | | | | | | | | | | | |
| 12.5 | E | | | | | | | | | | | | |
| 6.25 | F | | | | | | | | | | | | |
| | G | | | | | | | | | | | | NEG CONTROL |
| | H | | | | | | | | | | | | |

**Figure 3.6 B. 96 well microtiter plating schematic for MIC testing 12-way, with model 100i with sodium hypochlorite.**

Plating and incubation of bacteria into prepared 96 well microtiter plate:

At each predetermined time point, as shown in Figure 3.1, 1ml of liquid was pipetted from each sample. Each sample was diluted 1:10 twice into 9ml of 0.9% sterile saline. The first dilution in each sample was vortexed and poured into a sterile petri dish. 50µl of this sample was pipetted into each well containing antimicrobial dilutions. Each sample was plated in technical triplicate rows on the 96 well plate as shown in Figure 3.7.

Each well, except the negative control, contained approximately $5x10^4$ to $5 \times 10^5$ cfu/ml. The last row contained no antimicrobial, this was used as the positive growth control. 50µl of sterile 0.9% saline was added to the negative control well (G12).

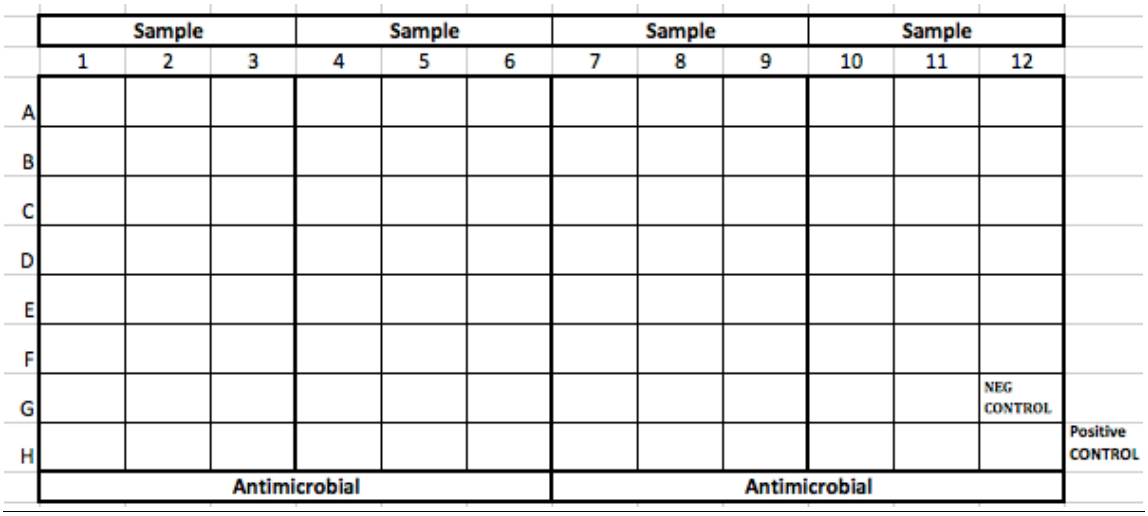


**Figure 3.7. 96 well microtiter plating schematic of bacteria for MIC testing with alternating current treatment compared to a control.**

The second 1:10 dilution from the test samples was used to sample enumeration of bacterial counts and plated as described above.

An alternating current technology using growth curves:

After inoculation, the 96 well plate was placed in an Epoch 2 microplate spectrophotometer (Biotek) programmed to read the optical density of each well at nm ($OD_{600}$) every 10 minutes for 18 hours at 37°C.

Determination of Signal Variability using three different alternating current units over two container Types in three different solutions:

The signal strength was measured using an Owon HDS102M oscilloscope on each of the three model units, HS48, 60i and 100i, over each of the testing parameters as shown in Table 3.1. Currents were measured around the ferrite ring, touching the outside of the container itself, and touching the top of the liquid substrate in the container.

**Table 3.1 Testing parameters performed over 4 different experiments.**

| Ferrite ring | Container | Solution |
|---|---|---|
| HS48 | 250ml glass Erlenmeyer flask | 0.9% saline |
| 60i | 250ml glass Erlenmeyer flask | 0.9% saline |
| 100i | Stainless steel container | BPW |
| 100i | Stainless steel container | Ion Salt Solution |

## Results:

Changes in alternating current frequency based on solution, container type and unit model

To determine the effect of different alternating current frequencies on *E. coli*, measurements were taken using an Owon HDS102M oscilloscope in order to establish the frequency in each system. Current output frequencies can be seen in Figure 3.8, frequencies can be seen in Table 3.2. Output for each unit is measured at 14-16v, 1.2 watts and 0.14A.

Each model produced a different alternating current frequency output when measured around the ferrite ring. Each frequency for the purposes of the results and discussion will be defined as its respective model number HS48 ACF, 60i ACF, and 100i ACF.

**Table 3.2 Alternating current frequency measurements across three models measuring the alternating current from the ferrite ring, container and solution.**

| Model | Factor | 1x vs 10x Probe | Time Difference | v~ | Vp | Frequency |
|---|---|---|---|---|---|---|
| HS48 | Ferrite Ring | 10x | 4.0ms | 500mv~ | 2.18v | 69.97 kHz |
| | Glass container | 10x | 4.0ms | 5.00mv~ | 3.60mv | 4.25 kHz |
| | 0.9% Saline Solution | 10x | 4.0ms | 200mv~ | 640.0mv | 52.61 kHz |
| 60i | Ferrite Ring | 10x | 4.0ms | 1 v~ | 5.92-7.6v | 72-73 kHz |
| | Glass container | 10x | 4.0ms | 50.0 mV~ | 276.0mv | 64.27 kHz |
| | 0.9% Saline Solution | 10x | 4.0ms | 5v | 16.2v | 42.92 kHz |
| 100i | Ferrite Ring | 10x | 4.0ms | 1v~ | 6.4v | 94-95 kHz |
| | Stainless Steel container | 10x | 4.0ms | 2v~ | 8.0v | 70-72 kHz |
| | BPW Solution | 10x | 4.0ms | 2v~ | 8.64v | 73 kHz |
| 100i | Ferrite Ring | 10x | 4.0ms | 1v~ | 6.96v | 94-95 kHz |
| | Stainless Steel container | 10x | 4.0ms | 2v~ | 10.16v | 70-72 kHz |
| | 0.3% Ion Solution | 10x | 4.0ms | 2v~ | 8.56v | 64.8 kHz |

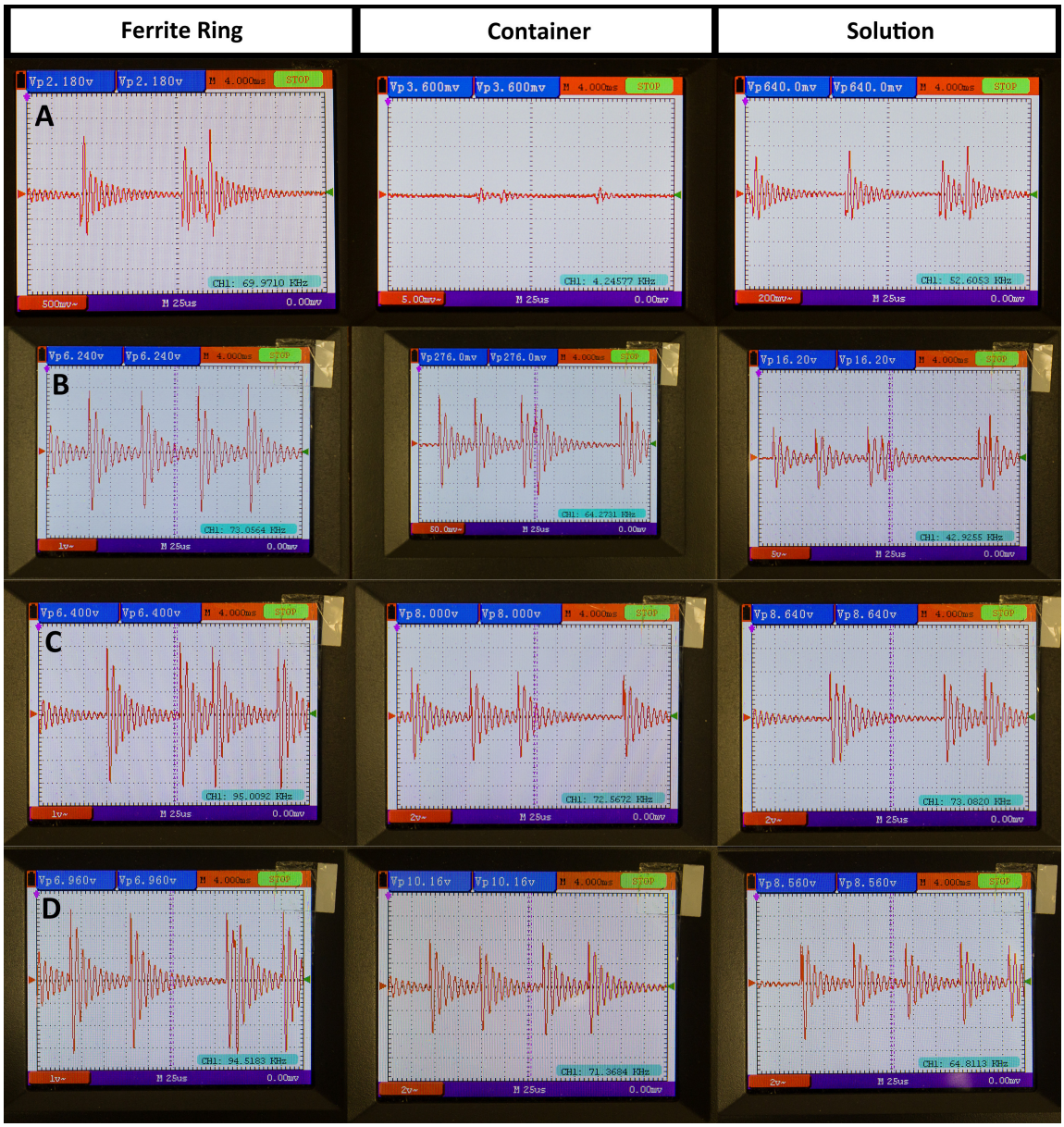


**Figure 3.8. Frequency readings using an Owon HDS102M oscilloscope measuring the alternating current output around the ferrite ring, the container and the top of the solution in each container. A. Model HS48 over a glass container containing 0.9% Saline. B. Model 60i over a glass container containing 0.9% Saline C. Model 100i over a stainless steel container containing BPW. D. Model 100i over a stainless steel container containing a 0.3% Ion solution.**

Statistical analysis of MIC results:

MIC growth curve data, at each time point of alternating current frequency (ACF) exposure, was compiled into Excel spreadsheets and analyzed based on defined optical density benchmarks. Each sample was measured for increased optical density over the 18 hours of testing. Benchmarks were defined as shown in Table 3.3. The initial benchmark chosen was an increase in optical density of 0.02. This was defined as the initiation of growth in each well. Each subsequent increase of 0.1, after the initial 0.02 benchmark, was defined as benchmark 2-6. The corresponding time point at each benchmark was converted into a decimal and analyzed.

**Table 3.3. Defined measurement of optical density increase for statistical analysis**

| Benchmark | Optical Density increase from Time 0 |
|---|---|
| 1 | 0.02 |
| 2 | 0.12 |
| 3 | 0.22 |
| 4 | 0.32 |
| 5 | 0.42 |
| 6 | 0.52 |

Statistical analysis was performed using a one-way ANOVA, Fisher's exact test, comparing Test and Control samples at each bench point. Statistical analysis was performed using the Statistica software analysis program.

Effect of models HS48 and 60i ACF on *Escherichia coli* O157:H7 in 0.9% saline in a static glass system exposed to Sodium Hypochlorite:

*E. coli* O157:H7 in a 0.9% Saline solution were exposed to either a model 60i or HS48 ACF for 6 and 24 hours. After removal from ACF exposure, bacteria were grown in minimal media containing either 100ppm or 50ppm Sodium Hypochlorite.
When bacteria were suspended in 0.9% Saline and exposed to ACF, there were no consistent statistical results when comparing across conditions for bacteria grown in a 50ppm solution (Data not shown). However, there were a larger proportion of significant results ($p < 0.05$) when bacteria were grown in a 100ppm solution. After the lower 6 hour exposure time, control bacteria took significantly longer to reach each benchmark in the 100ppm solution compared to the alternating current tests ($p < 0.05$), as seen in Figure 3.9. However, after the higher 24 hour exposure time, there was inconsistent significance toward the 60i ACF samples when compared to the control ($p < 0.05$). HS48 ACF results did not show statistical significance when compared to the control (Figure 3.10).

When comparing exposure times, bacteria exposed for longer periods showed slower optical density increase than the shorter time periods at each benchmark. When comparing frequencies, bacteria exposed to 60i ACF showed significantly ($p < 0.05$) lower growth rates compared to the bacteria exposed to HS48 ACF (Figure 3.11).

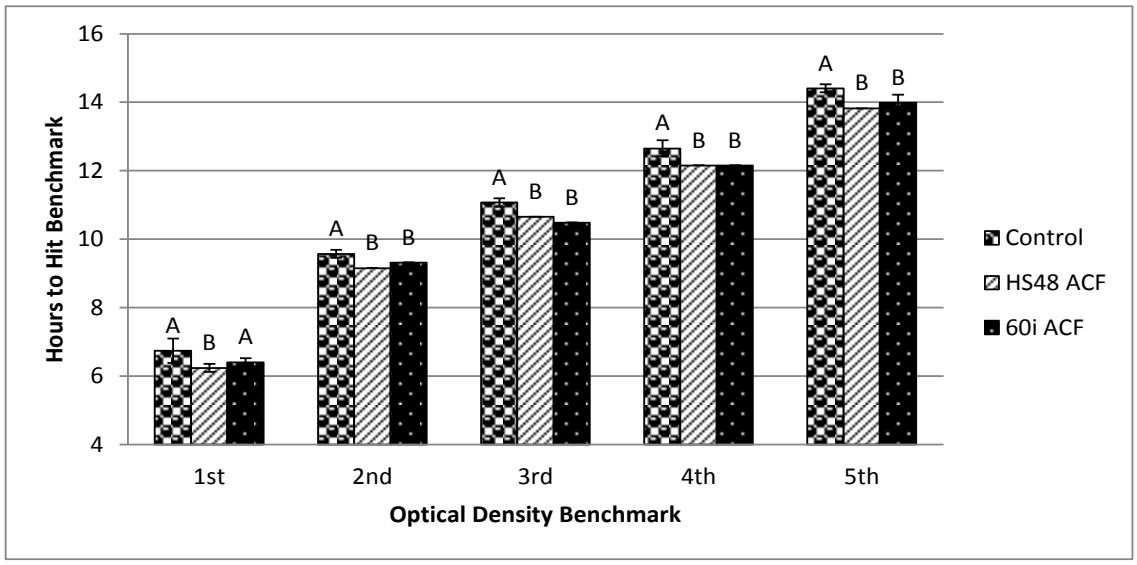


**Figure 3.9. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a 0.9% saline solution in a glass container while exposed to either a 60i or HS48 ACF for 6 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

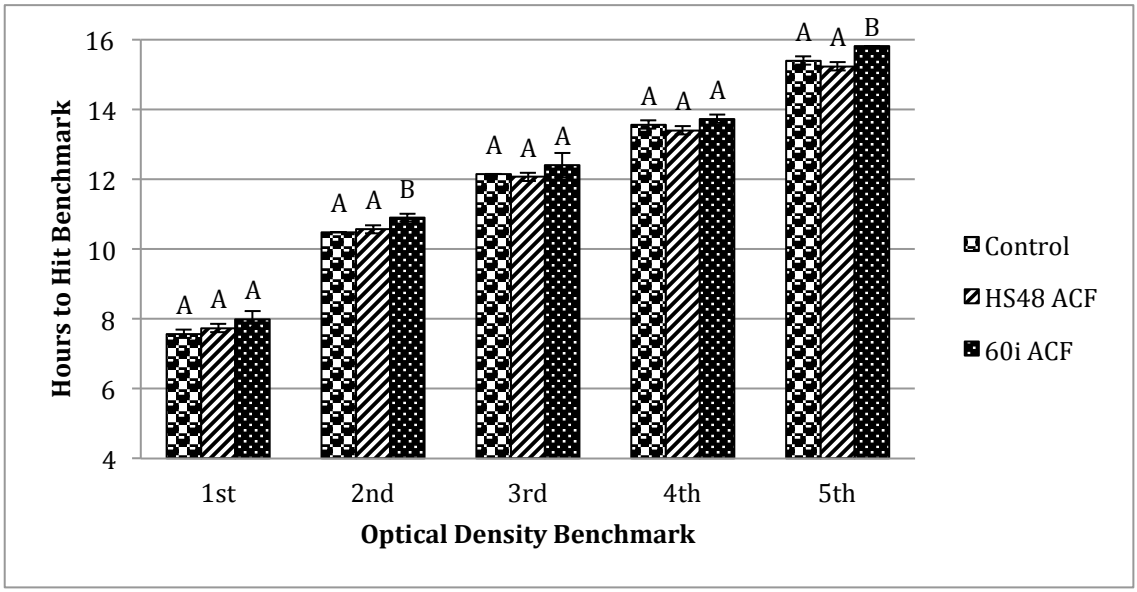


**Figure 3.10. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a 0.9% saline solution in a glass container while exposed to either a 60i or HS48 ACF for 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

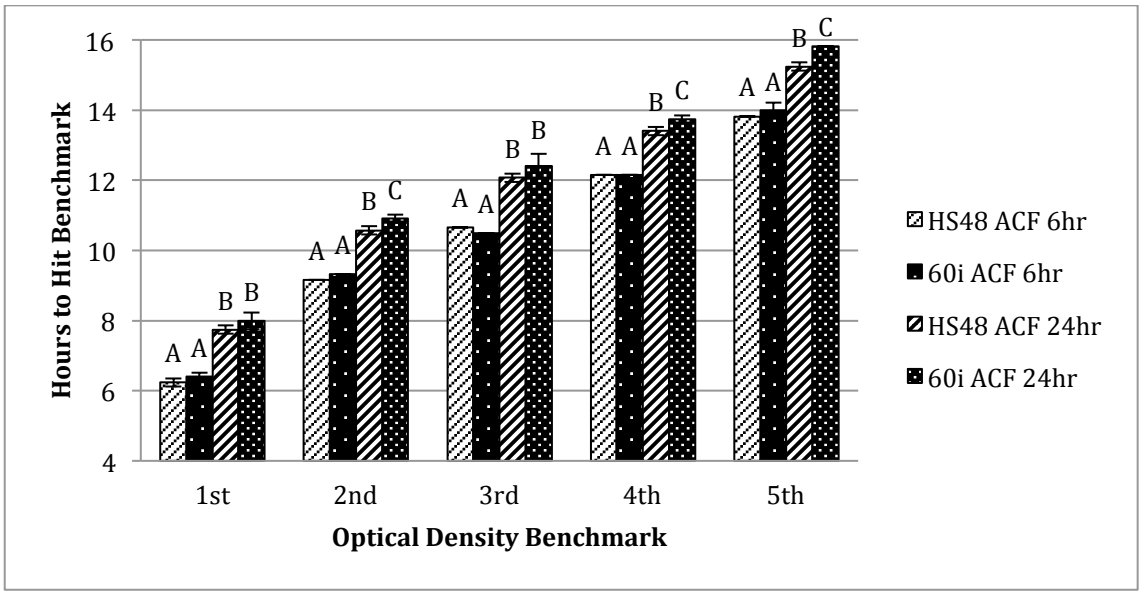


**Figure 3.11. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a 0.9% saline solution in a glass container while exposed to either a 60i or HS48 ACF for either 6 or 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

Effect of model HS48 and 60i ACF on *Escherichia coli* O157:H7 in BPW in a static glass system exposed to Sodium Hypochlorite:

*E. coli* O157:H7 in a buffered peptone water (BPW) solution were exposed to either a model 60i or HS48 for 6, 24 and 48 hours. After removal from ACF exposure, bacteria were grown in minimal media containing either 100ppm or 50ppm Sodium Hypochlorite. The only differences between this and the previous experiment are the use of a BPW solution instead of 0.9% Saline, and the addition of the 48 hour exposure. As in the previous test, bacteria grown in 50ppm media showed fewer and less consistently significant results ($p < 0.05$) when compared to those grown in 100ppm media.

When grown with 100ppm of Sodium Hypochlorite, bacteria exposed to the 60i ACF for 24 and 48 hours took significantly longer ($p < 0.05$) to increase optical density compared to control bacteria at all benchmarks (Figure 3.13 and 3.14). However, results were not significant when exposed for 6 hours compared to the control (Figure 3.12). Bacteria exposed to the HS48 ACF for 6 hours took significantly longer ($p < 0.05$) to reach benchmarks 1-3 compared to the control, but were not significant at the 24-hour exposure, and were only significant at the 2$^{nd}$ benchmark after 48 hours of exposure.

When compared across exposure times, bacteria exposed to the 60i ACF for 24 hours took significantly longer ($p < 0.05$) to reach optical density compared to those exposed for 6 hours at the 3$^{rd}$ and 4$^{th}$ benchmark. Bacteria exposed to 60i ACF for 48 hours took significantly more time ($p < 0.05$) to reach optical density when compared to those exposed for 24 hours at any benchmark. When comparing across frequencies, the 60i ACF showed consistent significance ($p < 0.05$) compared to the HS48 ACF after 24 and 48 hours of exposure but not after 6 hours as shown in Figure 3.15.

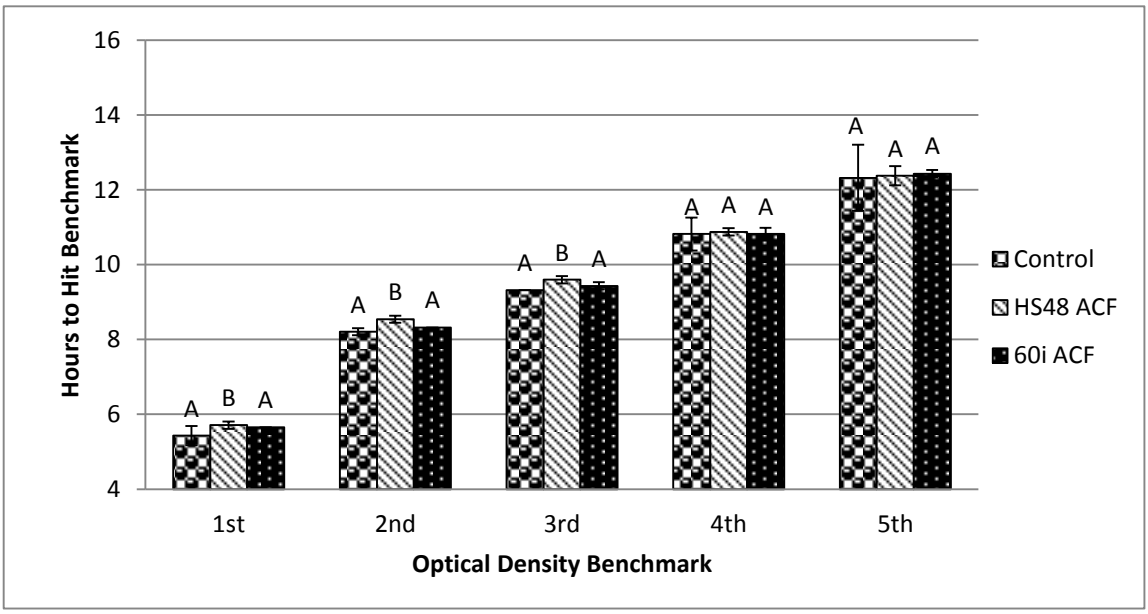


**Figure 3.12. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a 60i or HS48 ACF for 6 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

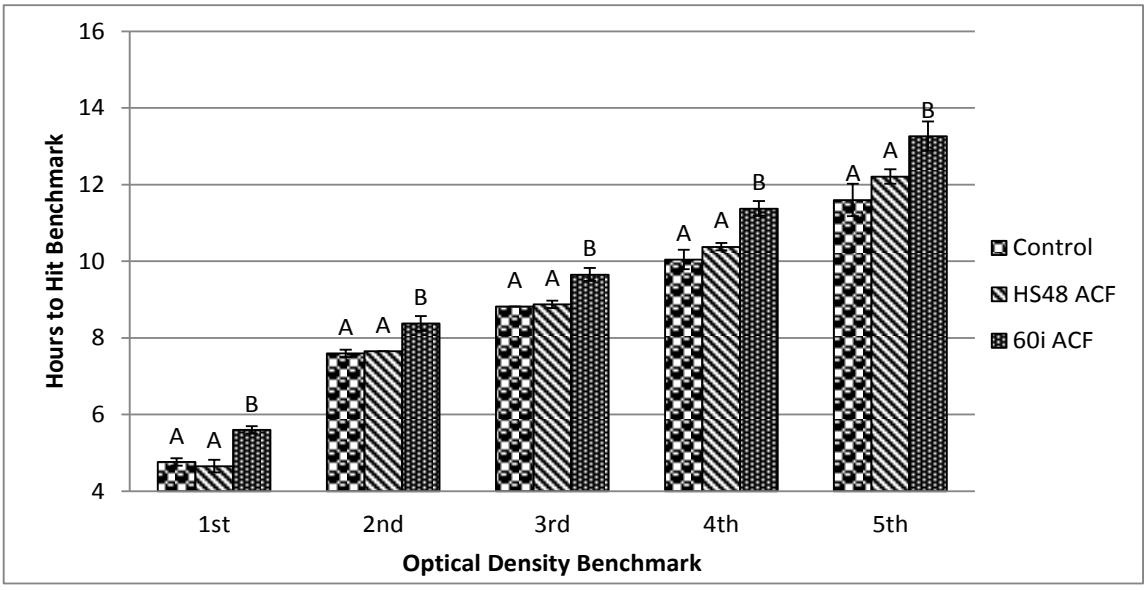


**Figure 3.13. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a 60i or HS48 ACF for 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

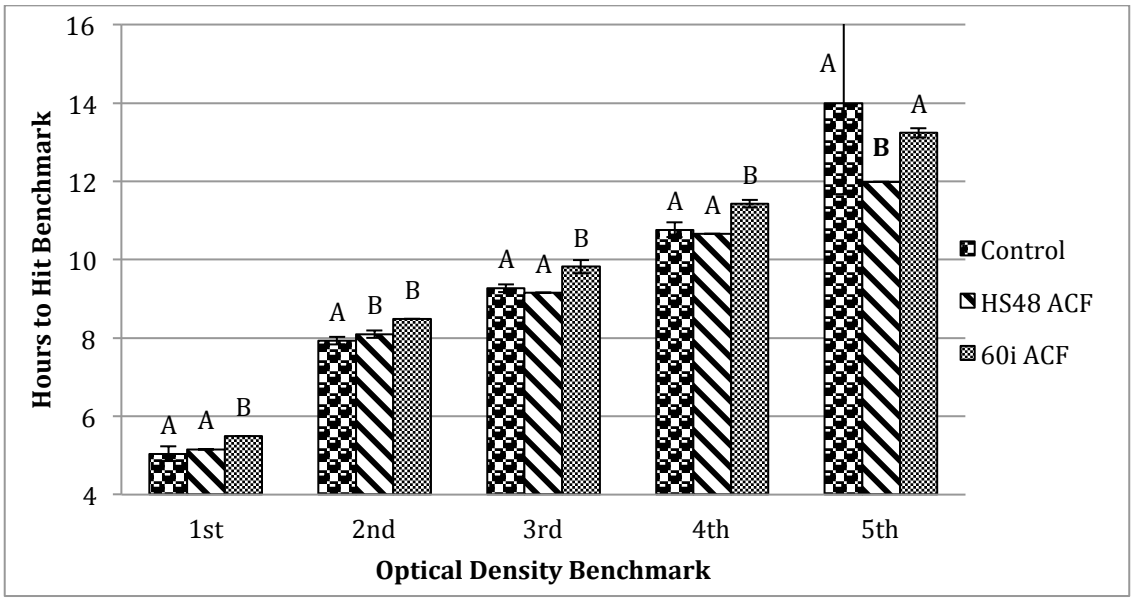


**Figure 3.14. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a 60i or HS48 ACF for 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

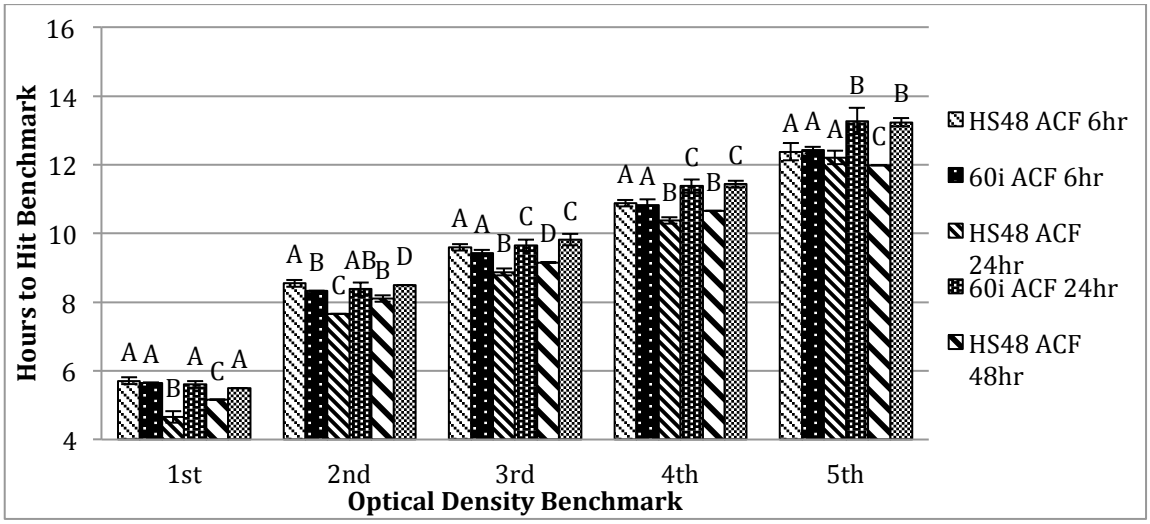


**Figure 3.15. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a 60i or HS48 ACF for either 6, 24 or 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

Effect of model 100i ACF on *Escherichia coli* O157:H7 in BPW in a static stainless steel system exposed to Sodium Hypochlorite:

*E. coli* O157:H7 suspended in a Buffered Peptone Water (BPW) solution were exposed to a model 100i ACF for 6, 24 and 48 hours. After removal from ACF exposure, bacteria were grown in minimal media containing either 100ppm or 50ppm Sodium Hypochlorite. The differences between this and the previous test are the use of the 100i ACF in place of the HS48 and 60i models and the static stainless steel system in place of glass.

When grown with 100ppm of Sodium Hypochlorite, bacteria exposed to the model 100i ACF for 6 hours took significantly longer ($p < 0.05$) to reach the 1$^{st}$, 2$^{nd}$, and 4$^{th}$ benchmark compared to the control as seen in Figure 3.16. Whereas bacteria exposed for 24 hours took significantly longer ($p < 0.05$) compared to the control at the initial benchmark but at no other benchmarks (Figure 3.17). Control bacteria took significantly longer ($p < 0.05$) to reach benchmarks 1-4 when compared to those exposed for 48 hours to the 100i ACF (Figure 3.18).

When exposure times were compared, bacteria exposed to the 100i took significantly longer ($p < 0.05$) to reach benchmarks after 6 hour exposure at benchmarks 1-5 when compared to the 24 hour exposure. Bacteria exposed for 24 hours took significantly longer ($p < 0.05$) when compared to bacteria exposed for 48 hours at benchmarks 1-4 (Figure 3.19). When grown with 50ppm of Sodium Hypochlorite, bacteria exposed to the

100i ACF for the shorter 6 hour time point took significantly longer ($p < 0.05$) to reach the 1st, 2nd and 4th benchmarks, when compared to those exposed for 24 hours.

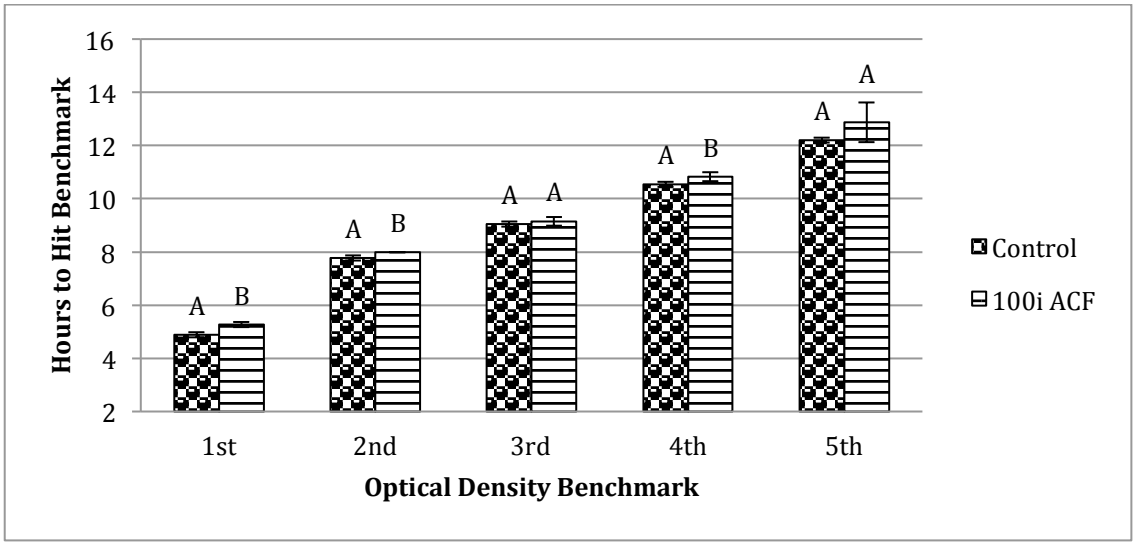


**Figure 3.16. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a stainless steel container while exposed to 100i ACF for 6 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

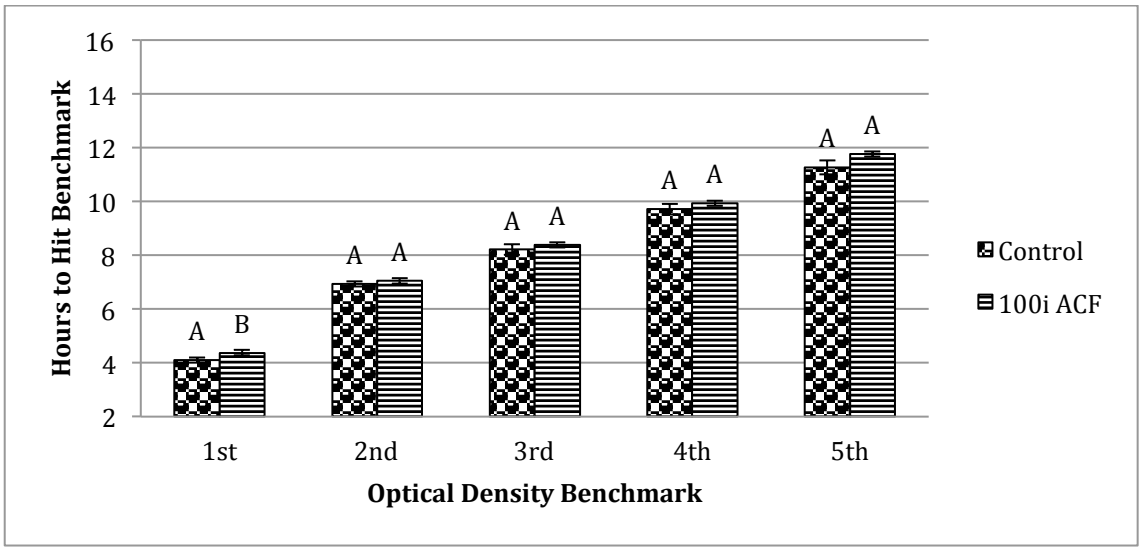


**Figure 3.17. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a stainless steel container while exposed to 100i ACF for 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

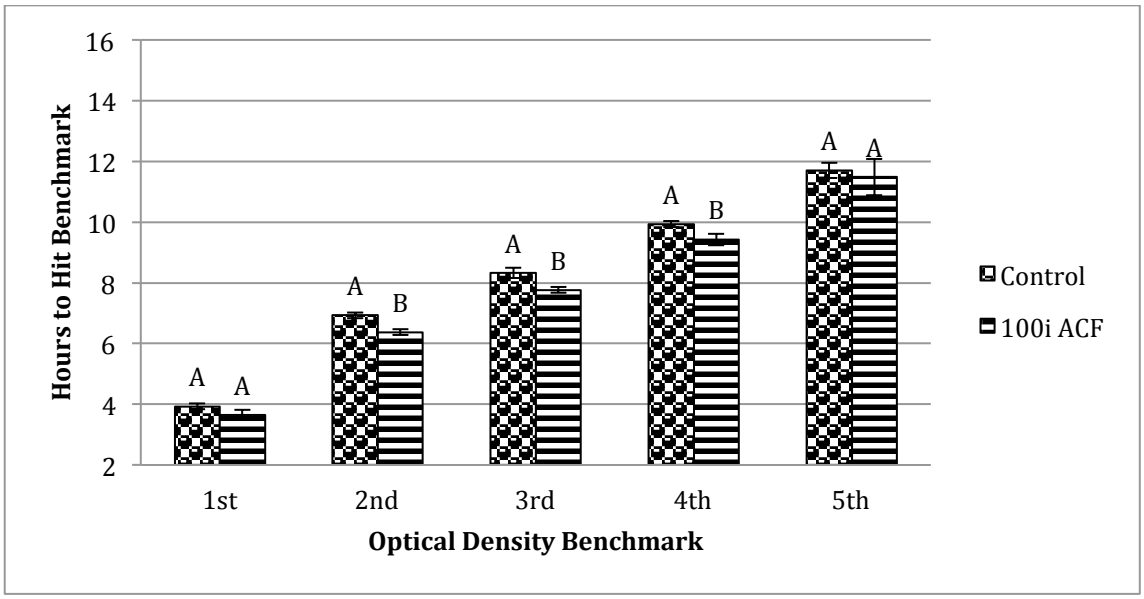


**Figure 3.18. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a stainless steel container while exposed to 100i ACF for 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

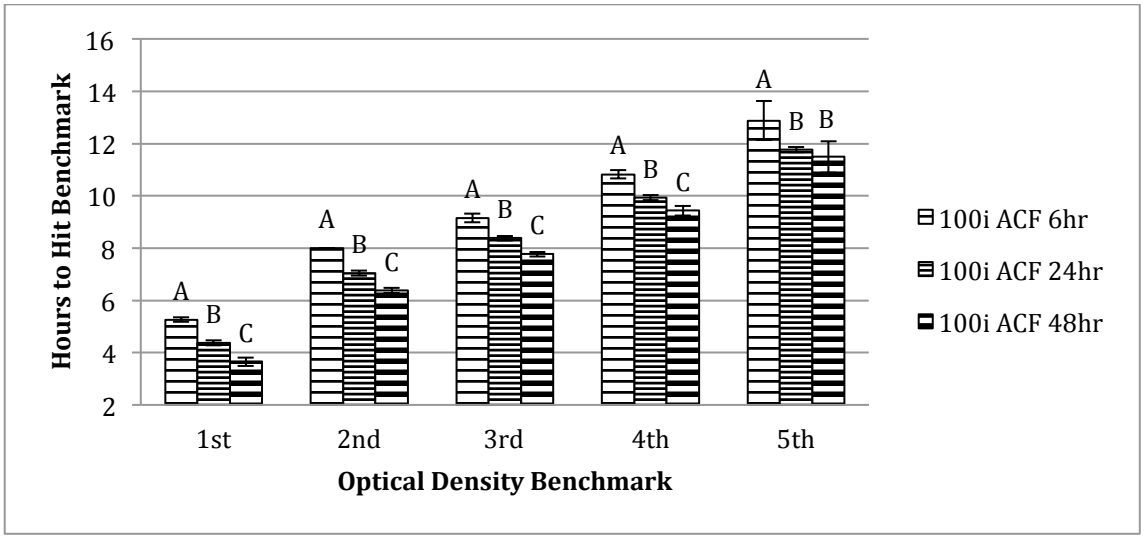


**Figure 3.19. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a stainless steel container while exposed to either a 60i or HS48 ACF for either 6, 24 or 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

Effect of model 100i ACF on *Escherichia coli* O157:H7 in 0.3% Ion salt solution in a static stainless steel system exposed to Sodium Hypochlorite:

Bacteria in a 0.3% ion salt solution containing $MgCl_2$, $CaCl_2$, KCl, and NaCl were exposed to a model 100i ACF for 6, 24 and 48 hours. After removal from ACF exposure, bacteria were grown in minimal media containing either 100ppm or 50ppm Sodium Hypochlorite. The difference between this and the previous test is the use of a 0.3% ion salt solution in place of the BPW solution.

When bacteria were exposed to 100i ACF for 24 hours and subsequently grown in the 100ppm media, the control took a significantly longer ($p < 0.05$) to reach benchmarks 1-5 (Figure 3.21). However, bacteria exposed for 48 hours took significantly longer ($p < 0.05$) to reach benchmarks 1-5 compared to the control (Figure 3.22). There were no significant results at the 6 hour exposure as shown in Figure 3.20.

When duration of exposure to 100i ACF was examined, bacteria exposed for 48 hours took significantly longer ($p < 0.05$) to reach optical density when compared to bacteria exposed for 24 hours for benchmarks 1-5. While bacteria exposed for 24 hours took significantly longer ($p < 0.05$) at only the $2^{nd}$ benchmark when compared to bacteria exposed for 6 hours (Figure 3.23).

When grown in 50ppm Sodium Hypochlorite, the control bacteria took significantly longer ($p < 0.05$) to reach the $1^{st}$-$3^{rd}$ benchmarks when compared to bacteria exposed to

100i ACF at both the 6 and 24 hour exposure times. Whereas bacteria exposed for 48 hours took significantly longer ($p < 0.05$) to reach the initial benchmark compared to the control, but no others.

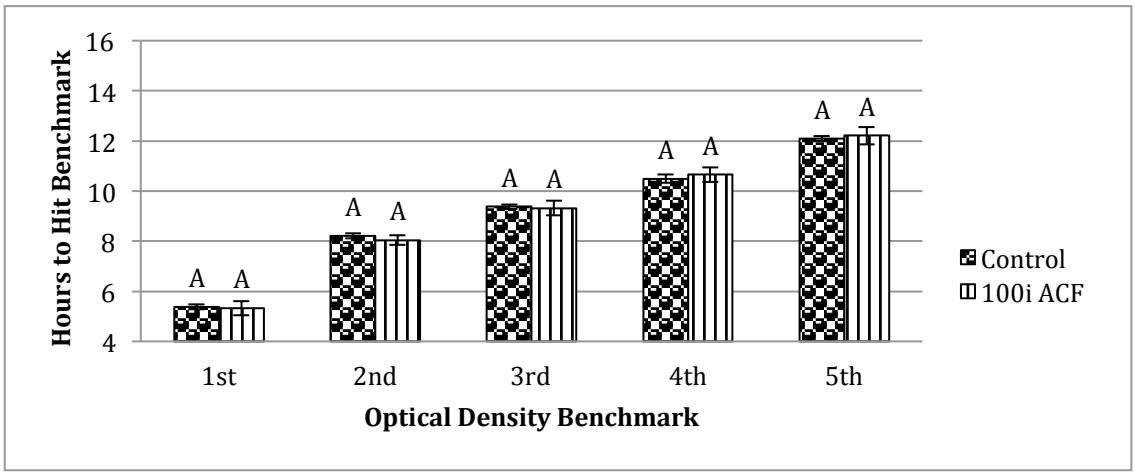


**Figure 3.20. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a 0.3% ion salt solution in a stainless steel container while exposed to 100i ACF for 6 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

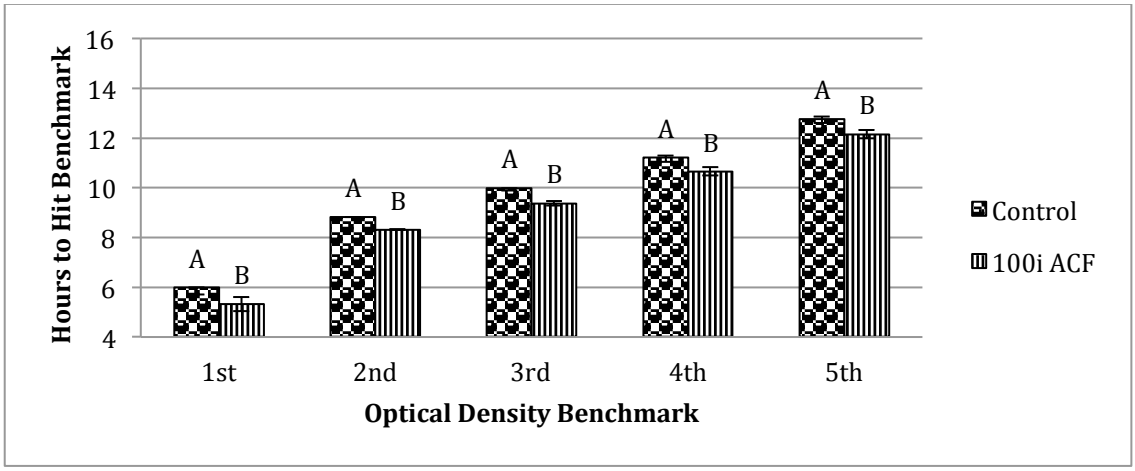


**Figure 3.21. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a 0.3% ion salt solution in a stainless steel container while exposed to 100i ACF for 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

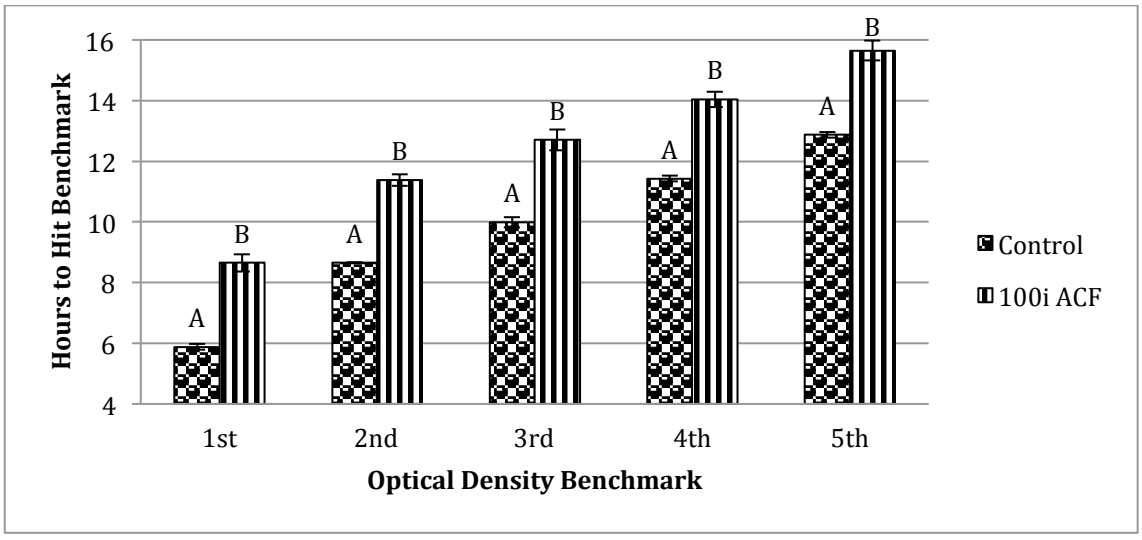


**Figure 3.22. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a 0.3% ion salt solution in a stainless steel container while exposed to 100i ACF for 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite, compared to a control.**

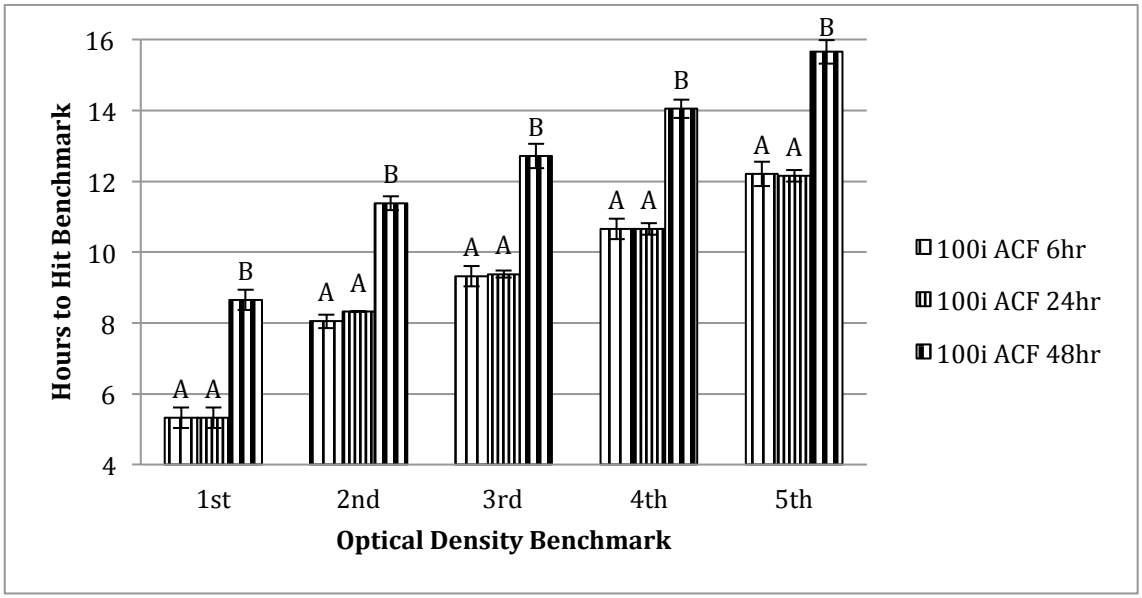


**Figure 3.23. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a 0.3% ion salt solution in a stainless steel container while exposed to either a 60i or HS48 ACF for either 6, 24 or 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

Effect of model 60i or HS48 ACF on *Escherichia coli* suspended in 0.9% Saline compared to BPW:

Statistical analysis was done across two alternating current frequencies (60i ACF and HS48 ACF) as well as two suspension solutions (0.9% Saline and BPW). After removal from ACF exposure, bacteria were grown in minimal media containing either 100ppm or 50ppm Sodium Hypochlorite. This scenario takes previous 60i/HS48 test results and compares the 0.9% Saline test results with those from the BPW tests. No control comparisons are covered here, as they were included in previous results.

Bacteria exposed to 50ppm of Sodium Hypochlorite showed significance between conditions up to the $5^{th}$ benchmark compared to bacteria exposed to the 100ppm media, which showed significance ($p < 0.05$) only up to the $4^{th}$ benchmark between conditions.

When grown with 100ppm Sodium Hypochlorite, bacteria suspended in 0.9% Saline and exposed to 60i ACF took significantly longer ($p < 0.05$) to reach benchmarks 1-4 after both 6 and 24 hours of exposure compared to bacteria in BPW exposed to HS48 ACF. Bacteria in 0.9% Saline and exposed to HS48 ACF took significantly longer ($p < 0.05$) to reach benchmarks 1-4 after both 6 and 24 hours of exposure compared to bacteria in BPW exposed to 60i ACF.

When comparing bacteria exposed to either 60i or HS48 ACF, bacteria suspended in 0.9% Saline took significantly longer ($p < 0.05$) to reach benchmarks 1-4 after 6 and 24 hours of exposure compared to bacteria suspended in BPW as shown in Figures 3.24 and 3.25.

When grown with 50ppm Sodium Hypochlorite, bacteria suspended in 0.9% Saline and exposed to 60i ACF took significantly longer ($p < 0.05$) to reach benchmarks 1-5 after both 6 and 24 hours of exposure compared to bacteria in the BPW solution exposed to HS48 ACF. Bacteria in 0.9% Saline and exposed to HS48 ACF took significantly longer ($p < 0.05$) to reach benchmarks 1-5 after 6 hours of exposure and to reach benchmarks 1-4 after 24 hours of exposure when compared to bacteria in the BPW solution exposed to 60i ACF as shown in Figures 3.26 and 3.27.

When comparing bacteria exposed to 60i ACF, bacteria suspended in 0.9% Saline took significantly longer ($p < 0.05$) to reach benchmarks 1-5 after 6 hours of exposure and to reach benchmarks 1-4 after 24 hours of exposure, when compared to bacteria suspended in BPW. When comparing bacteria exposed to HS48 ACF, bacteria suspended in 0.9% Saline took significantly longer ($p < 0.05$) to reach benchmarks 1-5 after both 6 and 24 hours of exposure when compared to bacteria suspended in BPW. The significant results seen in the comparison of 60i ACF to HS48 ACF alternating current was possibly masked by the significance of the 0.9% Saline solution.

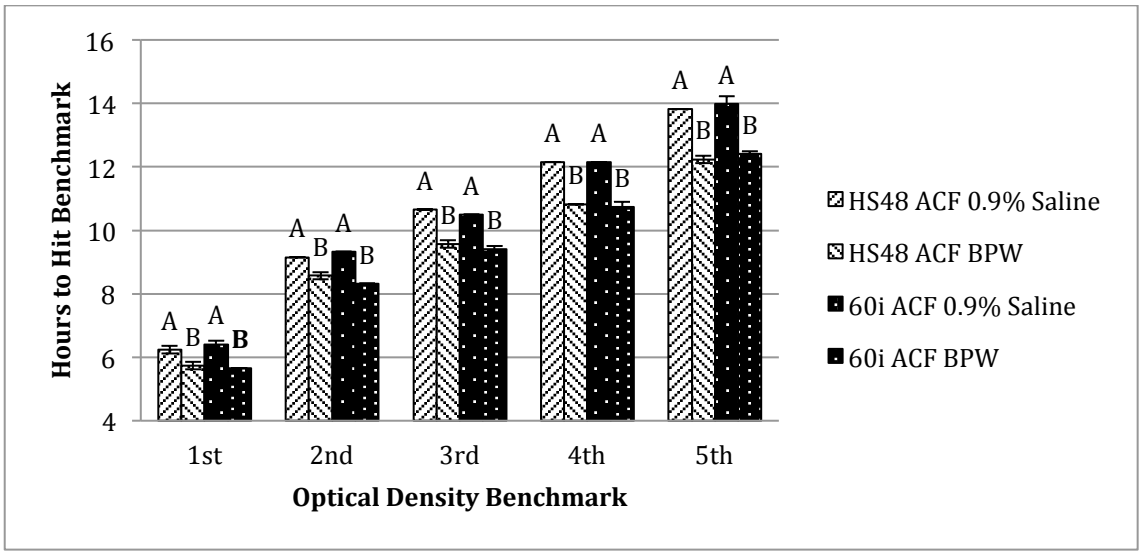


**Figure 3.24. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.9% saline solution in a glass container while exposed to either a 60i or HS48 ACF for 6 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

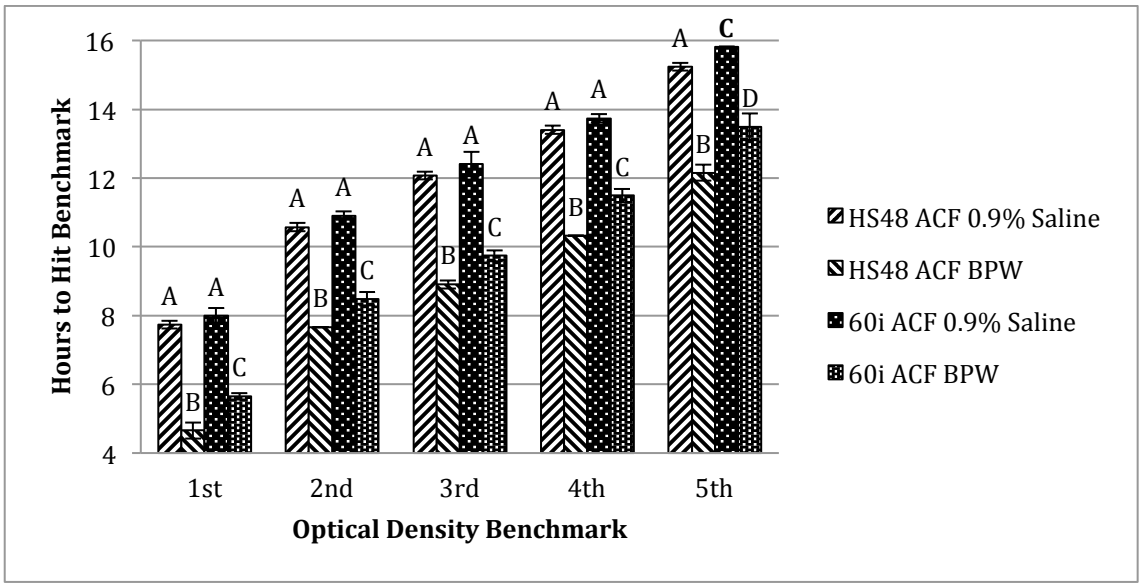


**Figure 3.25. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.9% saline solution in a glass container while exposed to either a 60i or HS48 ACF for 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

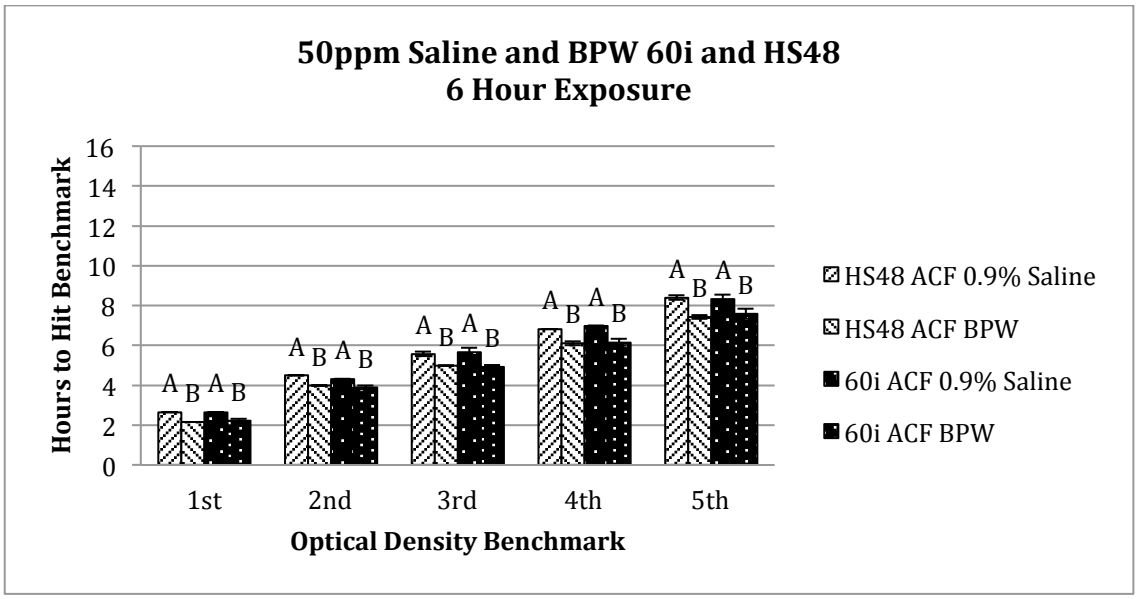


**Figure 3.26. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.9% saline solution in a glass container while exposed to either a 60i or HS48 ACF for 6 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

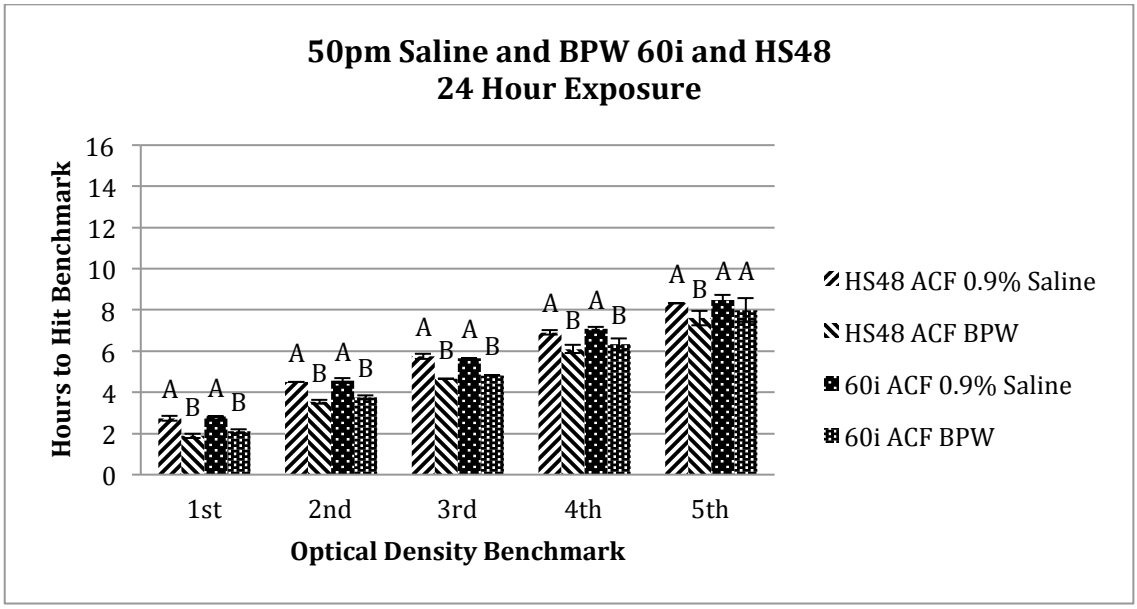


**Figure 3.27. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.9% saline solution in a glass container while exposed to either a 60i or HS48 ACF for 24 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

Effect of model 100i ACF on *Escherichia coli* O157:H7 suspended in a 0.3% Ion salt solution containing KCl, NaCl, $MgCl_2$, and $CaCl_2$, compared to suspension in BPW:

Comparison was done across two suspension solutions, BPW and a 0.3% ion salt solution, to determine their role in the efficacy of model 100i alternating current when bacteria are subsequently grown in a minimal media containing either 100ppm or 50ppm of Sodium Hypochlorite. This scenario takes previous 100i ACF results and compares those results from the 0.3% ion salt exposure tests with those from the BPW tests. No control comparisons are covered here, as they were included in previous results.

When grown in 100ppm media, results for bacteria suspended in a 0.3% ion solution were statistically significant ($p < 0.05$) when compared to bacteria suspended in BPW after 24 hours of exposure to 100i ACF at benchmarks 1-4 (Figure 3.29) and at benchmarks 1-5 after 48 hours of exposure (Figure 3.30). Results for bacteria suspended in BPW were significant ($p < 0.05$) when compared to bacteria suspended in a 0.3% Ion solution after 6 hours of exposure to 100i ACF only at the $5^{th}$ benchmark (Figure 3.28).

When grown in 50ppm media, results for bacteria suspended in a 0.3% ion solution were statistically significant when compared to bacteria suspended in BPW after 6 hours of exposure to 100i ACF at benchmarks 1-4 (Figure 3.31). While results for those same bacteria exposed for 24 and 48 hours were significant ($p < 0.05$) when compared to the BPW solution at benchmarks 1-5 (Figure 3.32 and 3.33).

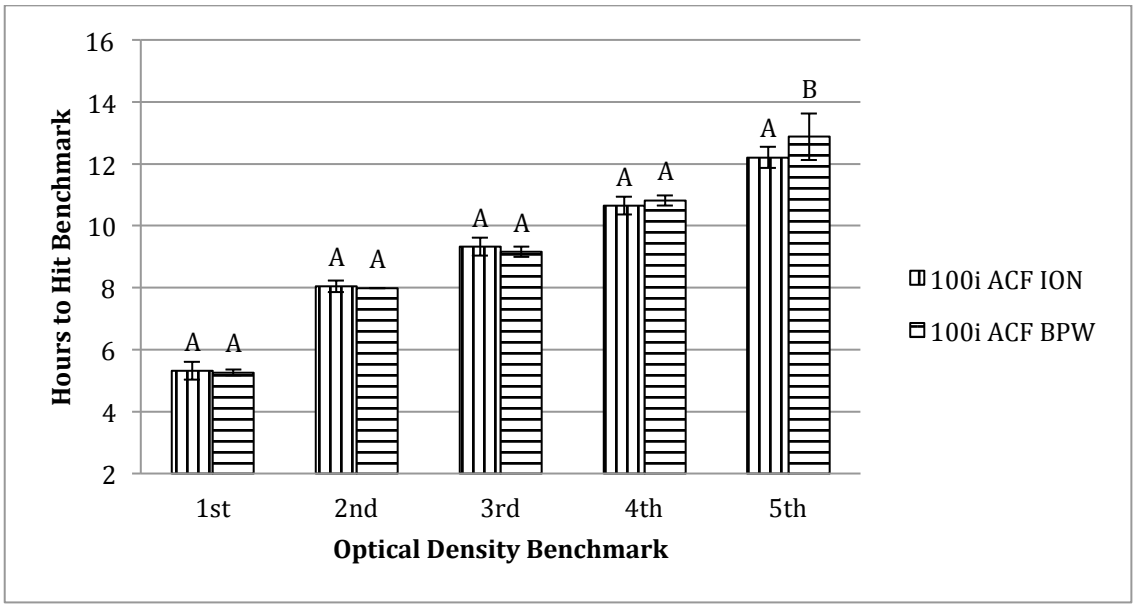


**Figure 3.28. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.3% ion salt solution in a glass container while exposed to 100i ACF for 6 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

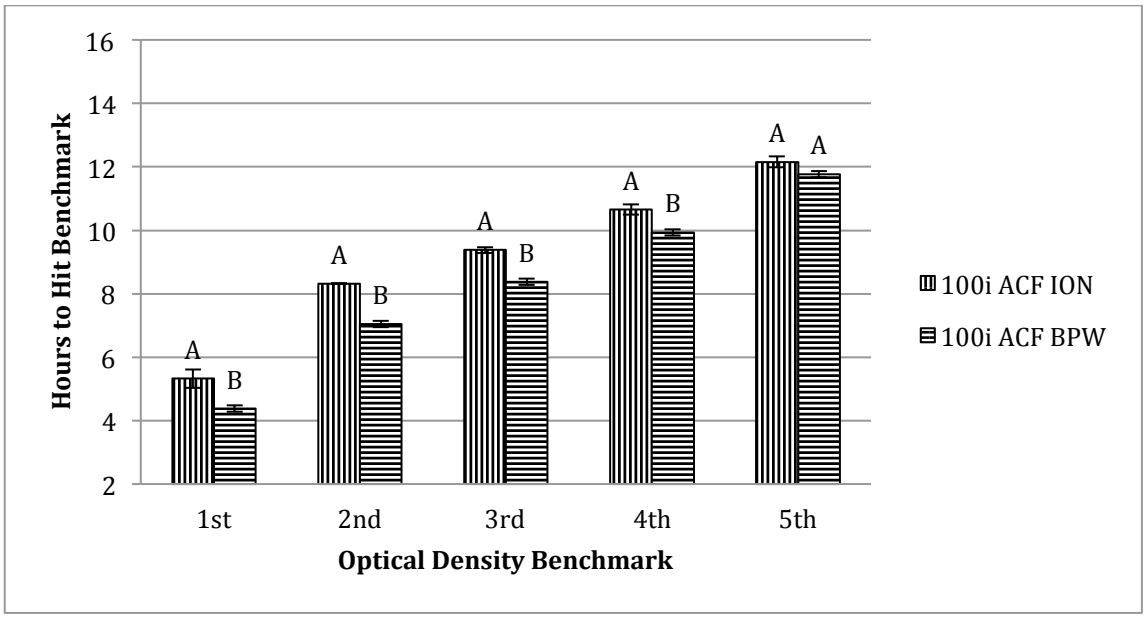


**Figure 3.29. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.3% ion salt solution in a glass container while exposed to 100i ACF for 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

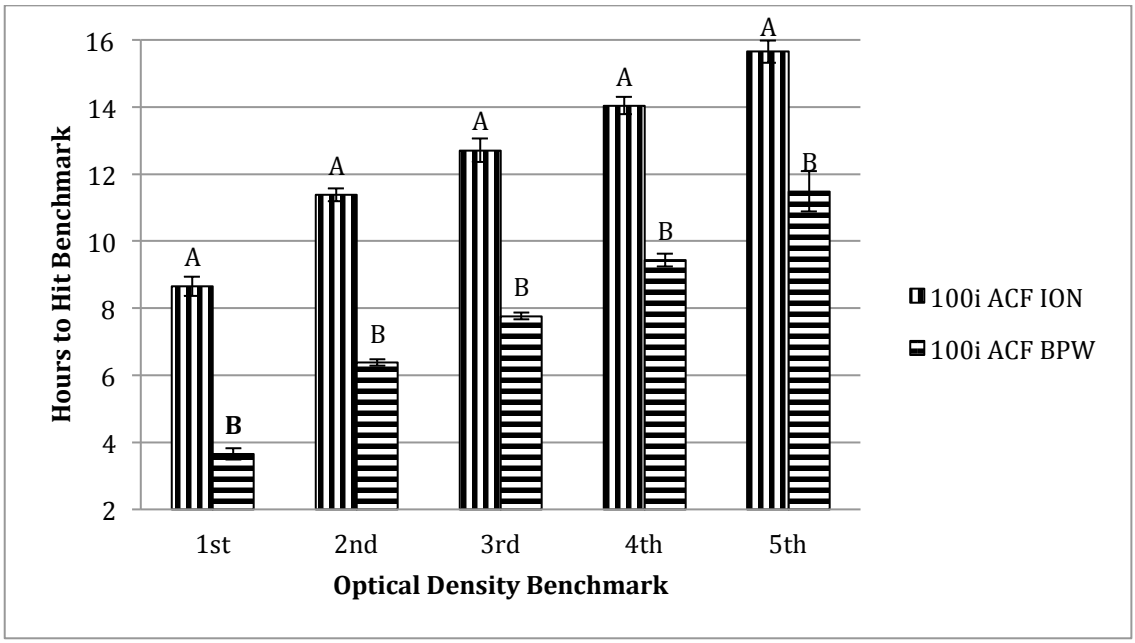


**Figure 3.30. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.3% ion salt solution in a glass container while exposed to 100i ACF for 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

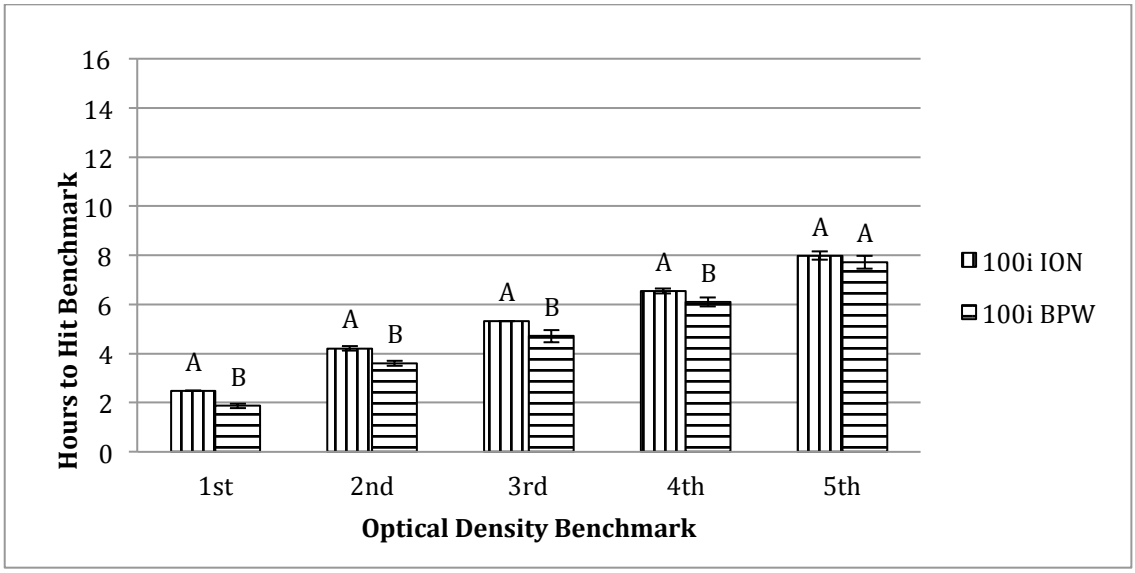


**Figure 3.31. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.3% ion salt solution in a glass container while exposed to 100i ACF for 6 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

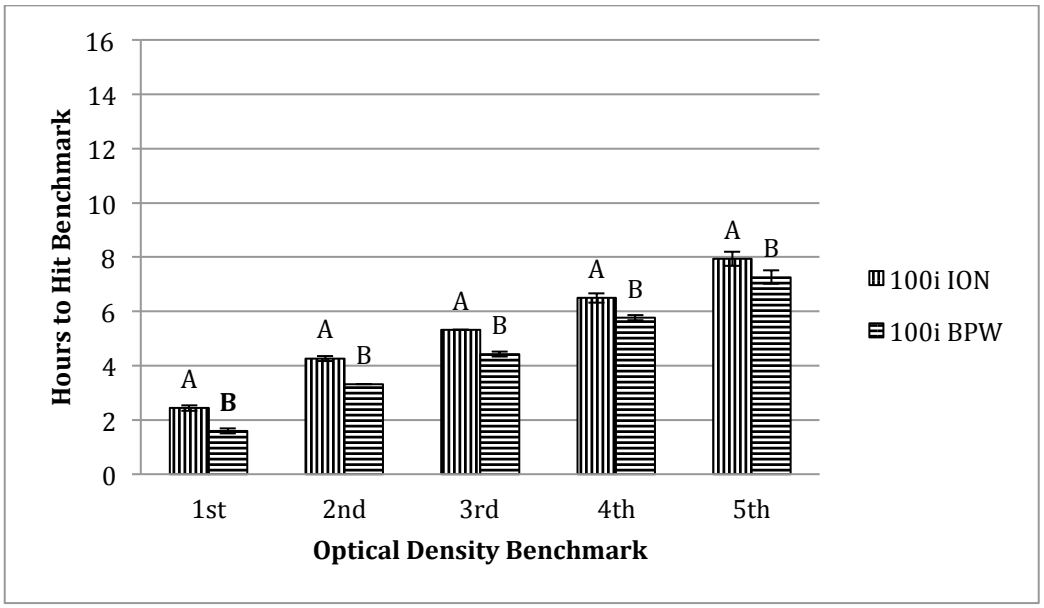


**Figure 3.32. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.3% ion salt solution in a glass container while exposed to 100i ACF for 24 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

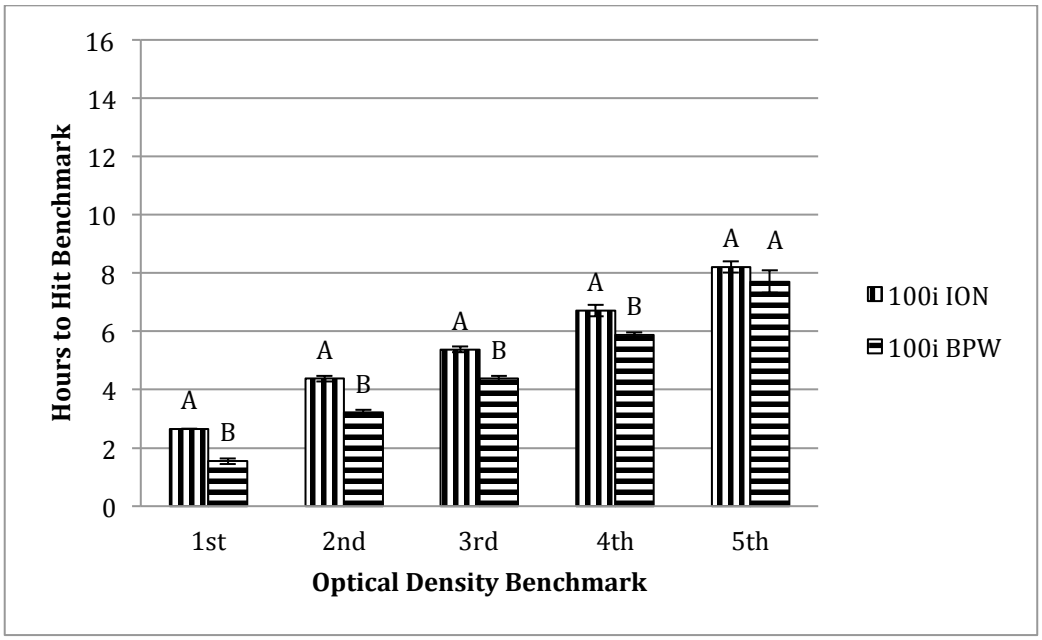


**Figure 3.33. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in either a BPW or a 0.3% ion salt solution in a glass container while exposed to 100i ACF for 48 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

Comparison of model 60i or HS48ACF in a glass system to a model 100i in a stainless steel system containing BPW:

Comparison was done across bacteria exposed to three ACFs (HS48, 60i and 100i) while suspended in BPW and subsequently grown in minimal media containing either 100ppm or 50ppm Sodium Hypochlorite. This scenario compares across previous BPW results for the HS48, 60i and 100i ACF. No control comparisons are covered here, as they were included in previous results.

When grown in 100ppm media, results for bacteria exposed to 60i ACF were significant ($p < 0.05$) after 24 and 48 hours of exposure at benchmarks 1-4 when compared to the HS48 (Figures 3.35 and 3.36); whereas bacteria exposed to HS48 ACF for 6 hours took significantly longer at the 2$^{nd}$ benchmark when compared to 60i ACF (Figure 3.34). Bacteria exposed to HS48 ACF for 6 hours took significantly longer ($p < 0.05$) to reach benchmarks 1-3 when compared to 100i ACF; whereas bacteria exposed for 48 hours were significant ($p < 0.05$) at benchmarks 1-4 when compared to 100i ACF as seen in Figure 3.36.

When grown in 50ppm media, bacteria exposed to 60i ACF for 24 hours took significantly longer ($p < 0.05$) to reach benchmarks 1 and 2 when compared to HS48 ACF (Figure 3.38). Bacteria exposed to HS48 ACF for 6 and 24 hours had significant results ($p < 0.05$) at benchmarks 1-3 when compared to 100i ACF (Figures 3.37 and 3.38), while bacteria exposed for 48 hours were significant ($p < 0.05$) only at the 1$^{st}$ and

2nd benchmarks (Figure 3.39). When compared to bacteria exposed to 100i ACF, bacterial growth exposed to 60i ACF was significant ($p < 0.05$) after 6 and 48 hours at the 1st thru 3rd benchmarks (Figures 3.37 and 3.39), but after 24 hours was only significant ($p < 0.05$) at the 1st and 2nd benchmarks (Figure 3.38).

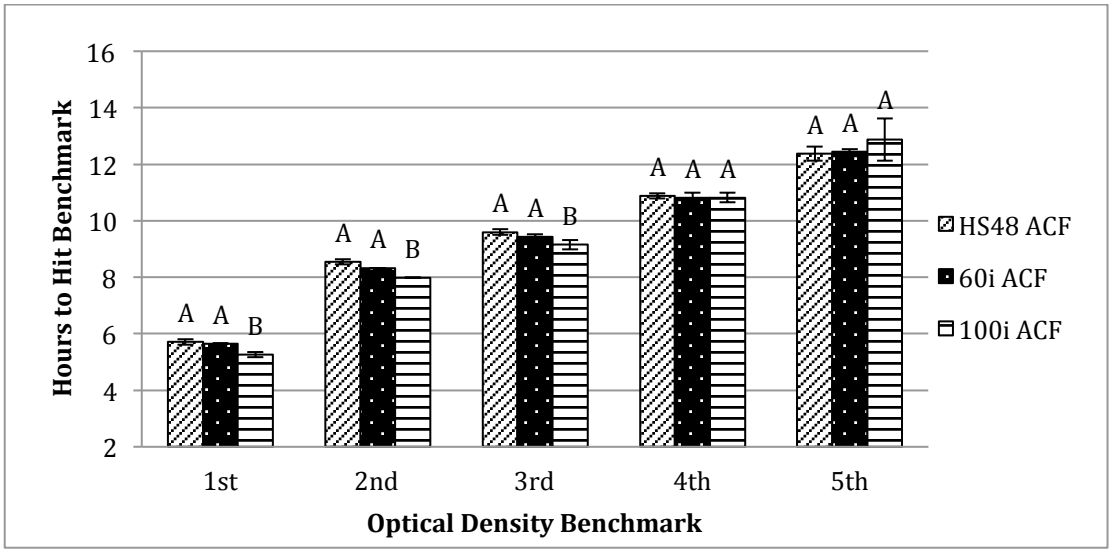


**Figure 3.34. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a HS48, 60i or 100i ACF for 6 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

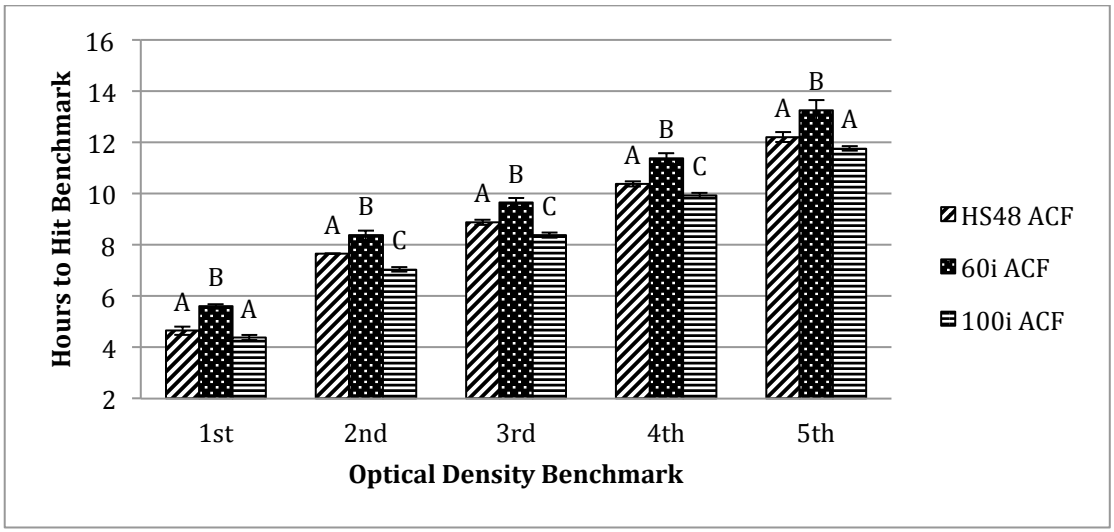


**Figure 3.35. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a HS48, 60i or 100i ACF for 24 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

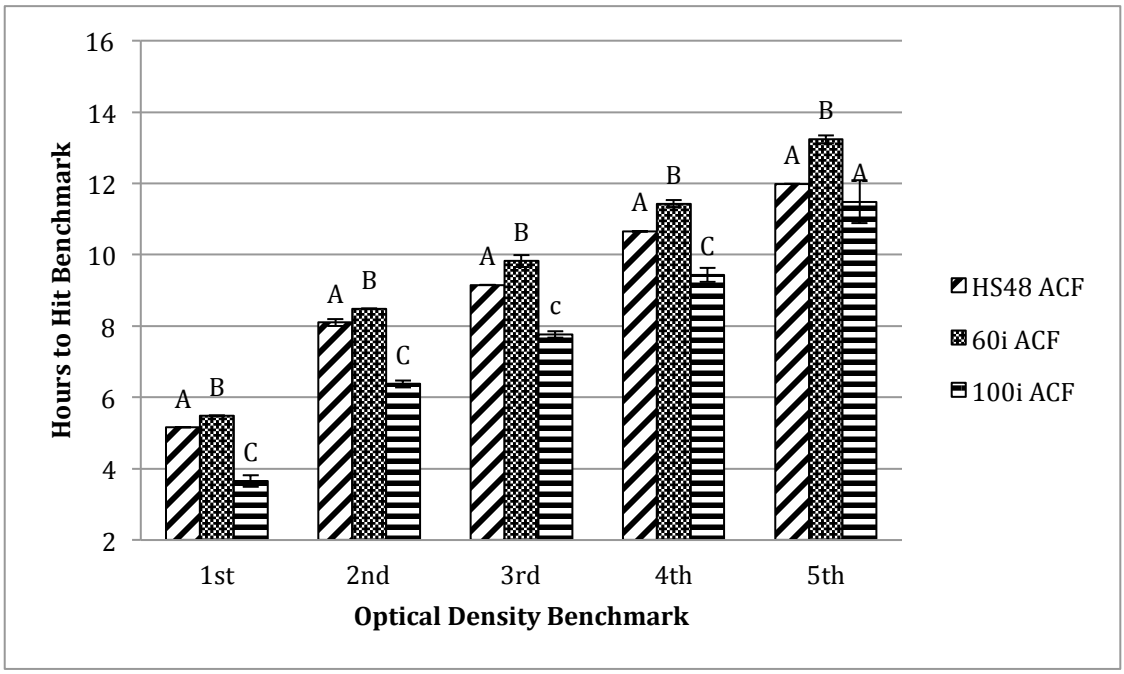


**Figure 3.36. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a HS48, 60i or 100i ACF for 48 hours and subsequently grown in minimal media containing 100ppm sodium hypochlorite.**

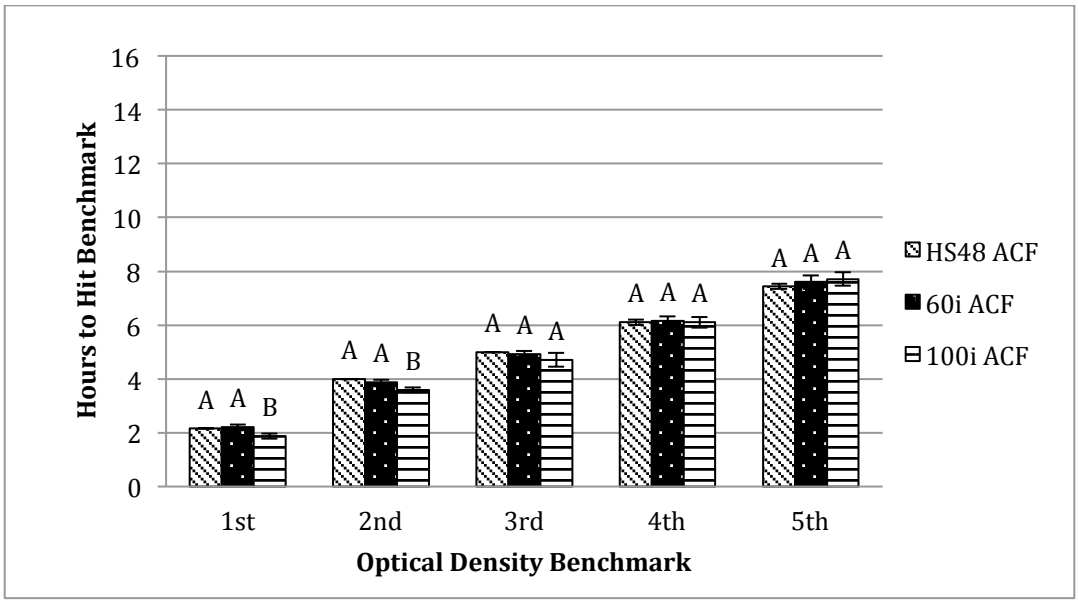


**Figure 3.37. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a HS48, 60i or 100i ACF for 6 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

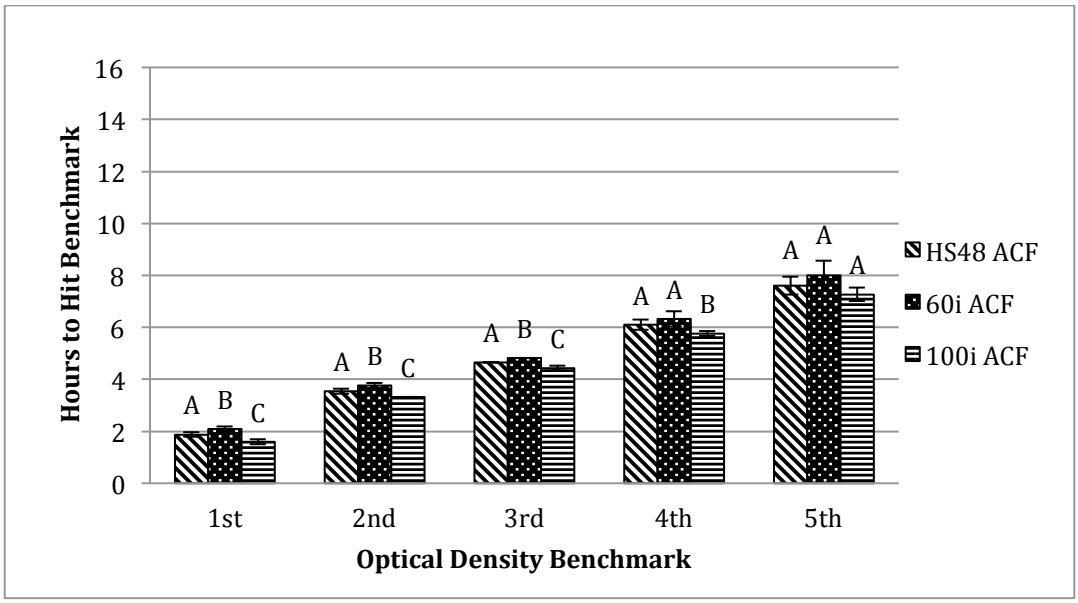


**Figure 3.38. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a HS48, 60i or 100i ACF for 24 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

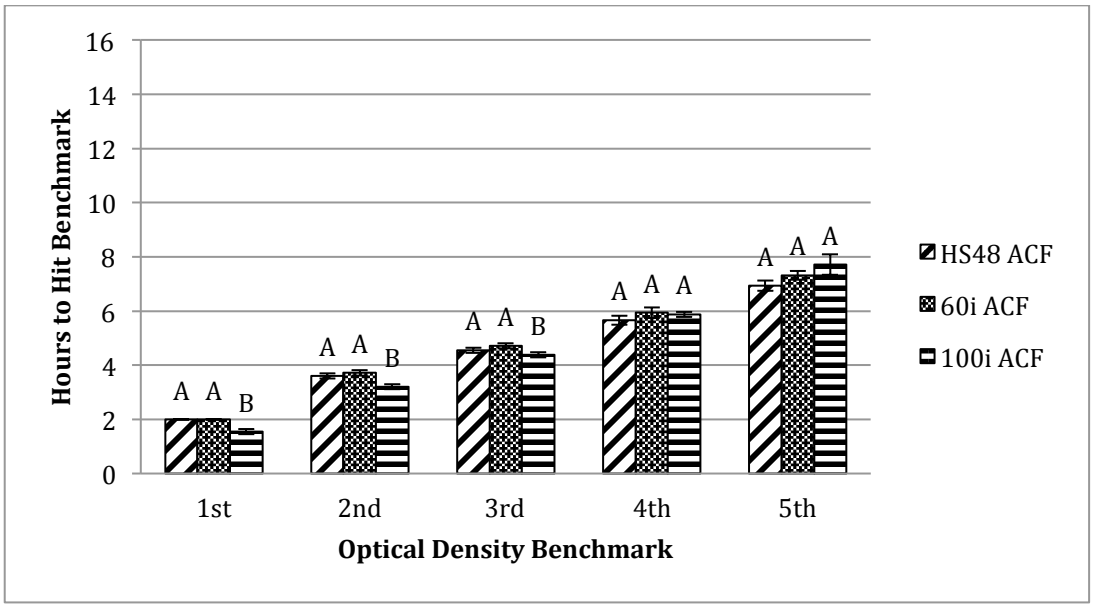


**Figure 3.39. Comparison of hours to reach optical density benchmarks when *Escherichia coli* O157:H7 was suspended in a BPW solution in a glass container while exposed to either a HS48, 60i or 100i ACF for 48 hours and subsequently grown in minimal media containing 50ppm sodium hypochlorite.**

Effect of 3 different models of alternating current frequency on bacterial counts in 0.9% NaCl, BPW or a 0.3% Ion Salt solution over 48 hours in either a glass or stainless steel container:

Samples were taken for enumeration at each time point to determine if there was a change in bacterial counts as well as to confirm the inoculum in each MIC well. There was no difference in bacterial counts of *E. coli* when compared to a control after 48 hours of exposure to alternating current (Data not shown).

## Discussion

This study investigated the effects of alternating current frequency (ACF) technology, as well as suspension solution, on the susceptibility of *Escherichia coli* O157:H7 when grown with varying levels of antimicrobial. This combination could be utilized to reduce bacteria in elevated bioburden solutions that would otherwise require high levels of antimicrobials to effectively control populations, reducing cost as well as reducing potential for harmful byproducts.

Optical density of bacterial growth media was measured; results were benchmarked and analyzed to determine the rate of increase of ACF treated bacteria compared to a control. Variables used included the alternating current frequencies, bacterial suspension solution, exposure time, container type and level of antimicrobial, as outlined in Table 3.1. Results were analyzed based on the time taken to reach each predetermined optical density benchmark. Comparison was done across ACF, exposure time, and suspension solution.

Across all bacterial suspension solutions exposed to ACF, there were few consistent significant results when comparing bacteria grown in solutions containing 50ppm of antimicrobial. However, there were a larger proportion of significant results ($p < 0.05$) when bacteria were grown in a 100ppm solution. When comparing exposure times, bacteria exposed to ACF for longer periods showed lower increase in optical density when compared to those exposed for shorter time periods at each benchmark. When

comparing frequencies, bacteria exposed to 60i ACF for longer exposure times showed lower growth rates compared to bacteria exposed to HS48 ACF, across most scenarios.

It was observed that control and test results showed fewer significant differences at the higher $5^{th}$ and $6^{th}$ benchmarks. This was found to be true across all tested scenarios - test and control bacterial growth levels tended to converge at the later benchmarks, within the 18 hour growth curve being tested. Therefore, it is the initial benchmarks that become the most important in determining the persistence of the ACF's impact on bacteria in solution, in order to determine the optimal exposure time as well as solution conditions for each ACF level.

When *Escherichia coli* O157:H7 is in a low saline solution, longer exposure time to 60i ACF is required to demonstrate significant delay in growth when exposed to 100ppm of Sodium Hypochlorite. *Escherichia coli* O157:H7 in a buffered peptone solution, while exposed to 60i ACF, took longer to reach benchmarks compared to the control after prolonged exposures and not after shorter exposure. In contrast, when *Escherichia coli* O157:H7 is in a buffered peptone solution exposed to 100i ACF, bacterial populations took longer to reach benchmarks after shorter exposure time. However, when *Escherichia coli* O157:H7 is in a 0.3% ion salt solution and exposed to 100i ACF, longer exposure time is needed to slow bacterial growth when grown in minimal media containing100ppm Sodium Hypochlorite.

Effect of model HS48 and 60i ACF on *Escherichia coli* O157:H7 in 0.9% saline in a static glass system exposed to Sodium Hypochlorite:

When grown in minimal media containing 100ppm of Sodium Hypochlorite, bacteria suspended in 0.9% saline took significantly longer to reach each benchmark after 24 hours of exposure to either 60i or HS48 ACF compared to bacteria exposed for only 6 hours. This suggests that longer exposure time to alternating current in a low salt solution causes an increased susceptibility when exposed to Sodium Hypochlorite. Those bacteria exposed for the shorter 6 hours, took significantly less time to reach each benchmark when compared to the control. This is in accordance with Inhan-Garip *et al*., (2011), who showed that the bacteria had an adaptive response after 6 hours of exposure to low frequency electromagnetic field. However, these results also suggest that prolonged exposure to ACF increases susceptibility, as shown in Figure 3.11, contradicting this adaptive response claim. This is similar to what was shown in both Caubet *et al*., (2004) and Giladi *et al*., (2010), who showed significant results at 24 and 48 hours of exposure respectively.

Effect of models HS48, 60i and 100i ACF on *Escherichia coli* O157:H7 in BPW in either a glass or a static stainless steel system exposed to Sodium Hypochlorite:

Bacteria suspended in BPW took significantly longer to reach each benchmark after shorter exposure times to 100i ACF when compared to both the control and to the longer exposure times. After the longer 48 hour exposure time, control bacteria took significantly longer to reach each benchmark compared to alternating current treated bacteria. See Figure 3.18. These results align with those seen using HS48 ACF, but are

in contrast to the results seen using 60i ACF when compared to the control, as seen in Figures 3.13 and 3.14.

When comparing frequencies, bacteria exposed to 60i ACF took significantly longer to reach each benchmark after all exposure times compared to the 100i ACF, and after the 24 and 48 hour exposure times, when compared to the HS48. Whereas bacteria exposed to the HS48 showed significance at benchmarks 1-3 and 1-4 when compared to the 100i after 6 and 48 hours of exposure, respectively, but only at the initial benchmark after 24 hours.

However, these results are contradictory to what would be expected. As frequency increases for longer exposures, we would expect the susceptibility of bacteria to increase as well. This may show that 60i ACF is the optimal frequency for solutions with high protein content, as BPW contains 1% peptone, an enzymatic digest of animal protein. These experiments utilized BPW containing 1.0% Peptone, 0.5% Sodium Chloride, 0.35% Disodium Phosphate, and 0.15% Monopotassium Phosphate. The amino acids in the peptone could have a positive effect on the growth of the bacteria in solution, making it more susceptible to alternating current frequency (Kirson et al., 2004) as well as simulate field conditions. This may also show that stainless steel is not an optimal container system in combination with the ACF, regardless of frequency strength.

The inhibitory effect of TTfields (150 kHz) has been attributed to two distinct mechanisms. First, interface with the formation of mitotic spindle microtubules, second, physical destruction of cells during cleavage. Both mechanisms are greatly dependent on the orientation of the axis versus the field vectors during mitosis (Kirson *et al*., 2004). This study demonstrated that an alternating electric field of 100 to 200 kHz interfered with the orientation of the spindle microtubules and the polymerization-depolymerization development involved in the chromosome separation process. Dividing cells affected by the electric current were also found to be oriented in the direction of the applied electric field. This would lead us to believe that under growth conditions promoted by the BPW solution, dividing cells would be more susceptible to the antimicrobial as was shown in Giladi *et al*., (2008). However, when compared to those bacteria suspended in ion salt solutions, BPW performed worse across all time points.

It is important to note that a direct comparison cannot be done between bacteria exposed to the 100i ACF and those exposed to either the 60i or HS48 ACF, as bacteria were housed in a stainless steel container when exposed to the 100i ACF and in a glass container when exposed to both the 60i and HS48 ACF.

Effect of model 60i or HS48 ACF on *Escherichia coli* suspended in 0.9% Saline compared to BPW:

When comparing increase in optical density across suspension solutions, bacteria suspended in 0.9% saline took significantly longer to reach optical density benchmarks

compared to bacteria suspended in BPW, independent of the ACF. As shown in Figures 3.24 thru 3.27**,** optical density results in 0.9% saline show a higher time point to reach each benchmark in both 100ppm and 50ppm Sodium Hypochlorite, compared to BPW samples. Therefore, any results for comparison of 60i ACF and HS48 ACF would be masked by the significance of the saline. This is not what would be expected after exposure to BPW, since the addition of peptone would aid in the growth of bacteria, allowing for disruption during cytokinesis (Kirson *et al*,. 2004, 2008), as discussed in previous sections. Here we observe the opposite effect, where those bacteria exposed to 0.9% saline are affected at each benchmark compared to the BPW. This could be due to lack of nutrients over time in a low ion solution, which may be increasing susceptibility of the cell to the antimicrobial.

Effect of model 100i ACF on *Escherichia coli* O157:H7 suspended in a 0.3% ion salt solution containing KCl, NaCl, $MgCl_2$, and $CaCl_2$, compared to suspension in BPW:

Comparison was done across suspension solutions (BPW and 0.3% ion salt solution), to determine their contribution to the efficacy of 100i AFC. It was observed that bacteria suspended in an ion salt solution took significantly longer to reach each benchmark compared to bacteria suspended in BPW across all comparable time points and dilutions, except for those exposed for 6 hours grown in 100ppm, as seen in Figures 3.28 thru 3.30. As described in previous sections, when comparing those bacteria suspended in 0.9% saline to suspension in BPW, the 0.9% salt solution also showed an increased time to reach each benchmark, as seen in Figures 3.24 thru 3.27. As described above, this is contradictory to the findings described by Kirson *et al*., (2004).

Frequency dependent inhibition of bacterial growth has been shown to relate to the effect of the alternating electric fields on the enzyme-substrate reaction equilibrium (Robertson and Astumian 1990; Giladi *et al*., 2008). This model suggests the electric charge distribution on some enzymes fluctuates with the conformational changes associated with enzyme-substrate interaction. Because the alternating current may affect molecular charge distributions, they could interfere with enzymatic reactions. The effect is expected to be larger for membrane enzymes, due to the membrane hindering the enzyme from rotating and escaping the effect of the electric field, as well as the fact the electric field is amplified in the membrane (Giladi *et al*., 2008). If essential enzymes are affected by the electric fields, exposure of these enzymes to certain frequencies may inhibit bacterial growth by exhausting the cell of the enzymes' products.

One explanatory protein found in *Escherichia coli* O157:H7 that is expected to be influenced by external electric fields is FtsK, which is a protein homologous to tubulin in eukaryotes and has been identified in all bacteria (Carballido-Lopez and Errington 2003). FskT is an essential cell division protein with a large dipole moment (Giladi *et al*., 2008), which is similar to the dipole moment of tubulin. Tubulin's structure and function has been demonstrated to be disrupted by alternating electric fields during mitosis (Kirson *et al*., 2004, 2007), as described above.

# Chapter 4:
# Conclusions and Future Work

We were able to show a preliminary difference in the effect of the alternating current when exposed to antimicrobials, as well as develop new methods for testing field applicable conditions in a lab setting, a task that had not been able to be executed in the past. Further study can now be done using these data as a stepping-stone to develop new methods that can help to validate an alternating current technology in a laboratory setting.

We were unable to design an effective model for testing an alternating current technology on a bench scale in a flow system. However, a modified minimal inhibitory concentration (MIC) method was designed for combining antimicrobials with the ACF technology to determine the enhanced effect on the bacterial cell after it is removed from the ACF and exposed to an antimicrobial.

When compared across the three alternating current frequencies, 60i ACF in a glass container was the optimal frequency to delay bacterial growth when bacteria were suspended in a buffered peptone solution. Test bacteria suspended in a salt solution, regardless of alternating current frequency, showed slower growth compared to bacteria suspended in a buffered solution when grown in minimal media containing either 100ppm or 50ppm Sodium Hypochlorite.

Future methods can include those described in Inhan-Garip *et al*., (2011) where samples were taken every hour to determine a growth curve when bacteria was suspended in a growth media investigating extremely low frequency electromagnetic fields over 6 hours. This method can be incorporated with different dilutions of antimicrobial, with and without electric current, to determine the effect of electric current on bacteria while exposed to antimicrobial, instead of the latency effect as explored in this study. Different suspension solutions as well as industry relevant antimicrobials can be utilized to determine the optimal frequency and suspension load in which the alternating current frequency can best perform.

Comparison studies can be done to determine this alternating current technology's effect in a lab setting as well as in field conditions. Using similar systems with both Gram positive, which would have more susceptibility to this treatment, and Gram negative bacteria, determination of the efficacy of the different alternating current frequencies can finally be established.

# Literature review


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